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## Habilitation à diriger les recherches

Aude Remot

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## HABILITATION À DIRIGER DES RECHERCHES

**En Sciences de la Vie et de la Santé**

**Année universitaire : 2022 / 2023**

présentée et soutenue publiquement par :

Dr Aude Remot

le 12/12/2022

-----

### **JURY :**

- Mme Caroline DEMANGEL	Directrice de recherche	Institut Pasteur Paris.
- Mme Isabelle DIMIER-POISSON	Professeure des Universités	Université de Tours
- M. Ed LAVELLE	Professeur	Trinity College Dublin, Irlande
- M. Kieran MEADE	Professeur	University College Dublin, Irlande
- Mme Nathalie WINTER	Directrice de recherche INRAE	Université de Tours
- Mme Véronique WITKO-SARSAT	Directrice de recherche INSERM	Université Paris Cité

Aux étudiants avec qui j'ai travaillé,  
merci pour ce bout de chemin ensemble.

A mes collègues,  
je suis chanceuse de venir travailler à vos côtés tous les jours.

#Waouh

## Remerciements

Ces remerciements seront tantôt en anglais, tantôt en français en fonction des personnes auxquels ils s'adressent, et comme je suis bavarde et bien entourée, ils sont un peu long.

First of all, I would like to warmly thank the five members of my jury, **Ed Lavelle, Kieran Meade, Caroline Demangel, Véronique Witko-Sarsat and Isabelle Dimier-Poisson**, who agreed to give their precious time to review my work. I contacted you because of your valuable expertise on immunology, mycobacteria and neutrophils. I hope you will enjoy this manuscript and maybe learn something useful as well, paving the way for future interactions! Thank you in advance for the scientific discussions we will have together on December 12<sup>th</sup>; I am really looking forward to it.

Je voudrais ensuite adresser par écrit des remerciements chaleureux à trois femmes scientifiques qui ont cru en moi, qui m'ont accompagné et encouragé. Si j'écris ce manuscrit d'HDR c'est grâce à vous trois. Par ordre chronologique :

Merci à **Sabine Riffault**, ma directrice de thèse. J'ai adoré travailler avec toi pendant 3 ans ½ sur ce projet passionnant d'immunologie néonatale, merci d'avoir proposé un projet de thèse abouti, éligible pour le contrat CJS. Je garde de merveilleux souvenirs de ma thèse, et je me sais chanceuse d'avoir toujours reçu des réponses positives à mes demandes (de formations, de participation à des activités annexes, à des congrès...) et d'avoir pu bénéficier d'un environnement scientifique de qualité pour lancer ma carrière de jeune chercheuse.

Merci à **Muriel Thomas**, mon encadrante pour mon post-doctorat. Tu as accepté de te lancer dans l'aventure du microbiote du poumon dans une unité de microbiologiste de l'intestin ! Merci pour ta confiance et mon autonomie pour lancer ce sujet dans ton groupe. Ta vision de physiologiste et tes questions m'ont challengé et guidé. Je suis ravie de voir que cette thématique continue après mon post-doctorat. Je repense souvent aux discussions avec le thé du matin, et à tout ce que j'ai appris sur les bactéries, surtout celles qui boulotent le mucus !

Merci à **Nathalie Winter**, mon mentor depuis mon arrivée dans ISP en décembre 2015. Merci pour ton accueil et mon intégration dans le groupe. Ton temps est précieux et ton agenda de DU chargé, mais tu es toujours disponible pour discuter des résultats, relire et corriger mes écrits. Merci pour la façon dont tu manages le groupe, ta confiance et ton dynamisme. J'adore les PMTB, les séances de brainstorm dans ton bureau avec les mindmap pleines de couleurs sur le tableau véleda, quand tu nous fais « relever la tête du guidon » pour prendre de la hauteur, ou que tu laisses « reposer les pâtes à crêpes ». Tu nous dis souvent qu'on obtient rarement des retours quand on fait les choses bien, alors je te l'écris : j'apprends énormément à tes côtés, humainement et scientifiquement. ISP est une unité riche et stimulante, que tu mets en valeur par tout ton travail. Ta passion pour la science (et les mycobactéries et les neutrophiles) est communicative, tes retours sont toujours judicieux et constructifs, et c'est un plaisir de venir au laboratoire tous les jours pour relever de nouveaux défis ensemble. Merci de cultiver le #Waouh!



Mes chers collègues IBIR (je ne voulais pas hiérarchiser, donc c'est par ordre alphabétique) :

**Badreddine – Bad pour les intimes**, merci d'être venu renforcer la parité dans l'équipe, on en a toujours besoin ! Bravo pour tous les résultats côté souris, ça dépote comme on dit. Quel farceur derrière cet air calme... je tombe dans le panneau à toutes tes blagues. J'espère que tu seras encore parmi nous pour longtemps. Tu as le droit de me réclamer des cornes de gazelles tous les ans (c'est écrit !).

**Christophe**, merci pour ta rigueur et ton organisation au labo, ta gestion des gros dossiers comme les devis/commandes pour équiper les labos, le rangement des amorces (dans les -20°C et virtuellement sur nos ordinateurs), quel bonheur de retrouver une info en un clic sur tes fichiers excel ! Merci de tout ce que tu fais pour l'équipe.

**Emilie**, par où commencer ? L'efficacité redoutable sous le PSM (plus vite partie...) ? Les discussions scientifiques et philosophiques au café/thé ? Les mindmaps en couleur ? Les précieux conseils de maman avisée ? Les séances de jogging (à réactiver !) ? trop difficile de choisir et je ne peux pas tout lister... mais l'idée tu l'auras comprise, c'est que tu es merveilleuse au quotidien, et que c'est génial de travailler avec une collègue comme toi. #Waouh que dire de plus !

**Florence (FloC)**, merci de m'avoir accueilli à bras ouvert, merci de me faire confiance et de t'impliquer dans mes projets. Merci pour les inoc de myco à  $\pm 0,1$  cfu, les (longueeees) séries d'extraction d'ARN, le housekeeping du labo, tes entrées/sorties dans le L3... que ferais-je sans toi ? et dire que je participe au cache-cache du chocolat...

**Florence (G)**, merci pour ton aide pour toutes les bactériologies sur le WP2 du projet Masticells et tes conseils pour Marion. J'adore aussi quand tu râles (99% du temps), on ne s'ennuie jamais avoir toi.

**Kamila and Pablo**, Obrigada pelo seu bom humor todos os dias, bom pós-doc! I must confess I 'google translated' the previous sentence so I hope you will get it! It is great to have you around, thanks for the Brazilian sunshine every day.

**Marion**, je ne vais pas déjà écrire tout le bien que je pense de toi ici, pas de copier-coller pour mon speech du 15 décembre ! Quel bonheur d'avoir partagé cette aventure de thèse avec toi. Je pense que scientifiquement parlant, on s'en est plutôt bien sorti (bravo pour toutes tes réussites, je suis très fière de nos articles). Merci pour ta rigueur, pour ton respect des deadlines, pour ton implication et ta motivation pour relever tous les challenges de cette thèse. Tu avais vraiment mérité ta semaine de manip Néolac ;-) On s'est bien amusé (ce n'est pas tout à fait fini !) et je garde les exemples pour le 15 décembre. Je te souhaite plein de réussites pour ton après-thèse, moi je ne suis pas encore prête pour mon après-Marion !

**Maryline**, merci pour ton sourire et ta gentillesse. Et merci aussi de gérer le Fluidigm, ce n'est pas une mince affaire !

**Nisha Tucker**, you are so much fun. I could listen to your lovely accent all day, we are lucky to have you in the team. I wish you all the best for your PhD, and I promise to be around if you need an extra hand to handle bovine cells – or just someone to go dance with (watch the knees)!

**Patricia**, merci pour les discussions au thé/café, merci pour ta gestion du labo de culture cellulaire et ce que tu fais pour l'équipe.

**Pascal**, merci pour tes conseils et la biblio immuno bovine que tu partages. Merci de continuer à t'impliquer dans l'équipe depuis ton départ en (presque) retraite.

**Pierre**, merci pour ton accompagnement, tes relectures et conseils. Depuis que tu as repris les rênes de l'équipe, la bonne ambiance règne dans les couloirs. C'est un challenge au quotidien, auquel tu accordes de l'importance, pour moi tu as relevé le défi. Merci pour ton aide pour les analyses avec R studio (un jour j'espère égaler le maître... j'ai encore beaucoup de travail !), pour ta participation aux manips, et vivement les challenges LPS (les vrais, pas les vacances en Grèce) ! J'ai failli oublier d'écrire « chef » pour te faire lever les yeux au plafond !

**Rodrigo**, merci pour la bonne humeur, l'entraide entre jeunes chercheurs, et de m'avoir montré le chemin pour le JCJC ! Merci d'avoir recruté de supers collaborateurs dans l'équipe, et de nous internationaliser par la même occasion ! Je te souhaite plein de réussite, pour CellBoVax et le reste, et j'espère qu'on aura des projets qui vont converger pour travailler ensemble après Masticells.

Après l'équipe IBIR, je voudrais ensuite faire cinq clins d'œil que me tiennent à cœur :

A **Delphyne**, à nos supers manips Krumdieck (surtout celles avec nos gros ventres de femmes enceintes qui touchaient les PSM). On papote ensemble autant qu'on dépote en manip ensemble (c'est presque prétentieux tellement on papote !), on s'est bien trouvé quand tu es arrivé à Jouy. Vivement la prochaine occasion de sortir une bouteille de Vouvray, ta maison en Touraine aura toujours ses portes ouvertes pour toi.

Une pensée spéciale pour **Sonia**, HealthyCalf, Métabov, et toutes les petites choses qui ne rentrent pas directement dans un projet... merci pour toutes ces interactions ! C'est toujours un plaisir. ISP a de la chance d'avoir une chercheuse comme toi.

**Yves (et Alix)** merci pour les tris de neutrophiles, pour la super viabilité, pour toutes les mises au point et l'aide sur nos panels. C'est toujours un plaisir de descendre vous voir, même quand les % de R6 ne sont pas géniaux !

**Steve**, you too are really amazing, and I also wish all my collaborators were like you! Thanks for welcoming me to UCD every year on different occasions, it is a true pleasure each time (I think about you whenever I have 1 cross -or more- on my hand). Ireland is becoming my 2<sup>nd</sup> home, people are so great that I invited two Irish researchers to review this HDR!

**L'équipe SAR au grand complet** : Chercheuse c'est aussi beaucoup de paperasse, alors merci à mes collègues du service d'appui pour leur aide sur les montages budgétaires, le partenariat, les contrats, les commandes, les procédures d'accueil, l'informatique... merci pour vos sourires, votre patience, votre réactivité et votre disponibilité.

Traditionnellement, le mot de la fin pour ma famille, qui ne comprend pas toujours ce que je fais mais comprend que ce travail est important pour moi. Merci de me faire relativiser. Merci à mon mari Kévin, qui assure tellement au quotidien que quand je rentre du travail je peux passer du temps avec mes filles au lieu de gérer l'intendance de la maison. Merci de comprendre que parfois j'ai besoin de travailler tard sur l'ordinateur, sans jamais me faire de reproche pour mes veillées solitaires, et désolé que les 346k€ de l'ANR JCJC n'arrive pas dans le budget pour notre maison ;-)

Gros bisous à mes deux filles, Maëlys et Léna, si un jour vous lisez « le travail de maman », je vous embrasse.

# SUMMARY

<b>1. List of Abbreviations</b> .....	1
<b>2. Introduction</b> .....	3
<b>3. Curriculum Vitae</b> .....	5
<b>4. Research activities</b> .....	9
A. Immunological signature of neonatal susceptibility to respiratory syncytial virus infection (PhD).....	9
i. Context and state-of-the-art .....	9
ii. Scientific questions .....	10
iii. Main results .....	11
iv. Related publications .....	13
B. Influence of lung microbiota on neonatal immunity (Post-doctorate).....	14
i. Context and state-of-the-art .....	14
ii. Rationale and hypothesis .....	15
iii. Aim and research objectives .....	15
iv. Main results .....	15
v. Related publications .....	17
C. Bovine innate responses in the context of major bacterial infections (INRAE researcher).....	18
i. Aims and research objectives .....	18
ii. Bovine tuberculosis: context and state of the art.....	18
iii. Deciphering the early lung responses to mycobacteria .....	22
iv. First characterization of bovine neutrophil subsets.....	25
v. Mastitis: context and state of the art.....	29
vi. Deciphering the role of neutrophils subsets in the context of clinical and subclinical mastitis (Masticells project and Marion Rambault's PhD project, 2019-2023).....	30
D. Grants.....	31
<b>5. Mentoring</b> .....	34
<b>6. Teaching and evaluation activities</b> .....	36
<b>7. Perspectives</b> .....	38
<b>8. Annexes</b> .....	42
A. Publications .....	42
C. Oral communications .....	49

D. Posters.....	51
E. Scientific communication to the general public .....	53
Selected publications	
PhD n°1. Remot et al., EJI 2011 .....	55
PhD n°2. Remot et al., PLoS ONE 2012.....	65
PhD n°3. Remot et al., EJI 2016 .....	78
Post-doc n°1. Remot et al., ISME J 2017 .....	89
Post-doc n°2. Mathieu et al., Frontiers Physiol 2018 .....	103
CRCN n°1. Remot et al., Frontiers Immunol 2019 .....	114
CRCN n°2. Rambault et al., Frontiers Immunol 2021 .....	124
CRCN n°3. Remot et al., Frontiers in Vet Science 2021 .....	138
<b>9. Summary in French - Synthèse en français .....</b>	<b>154</b>
<b>10. References .....</b>	<b>175</b>

## 1. List of Abbreviations

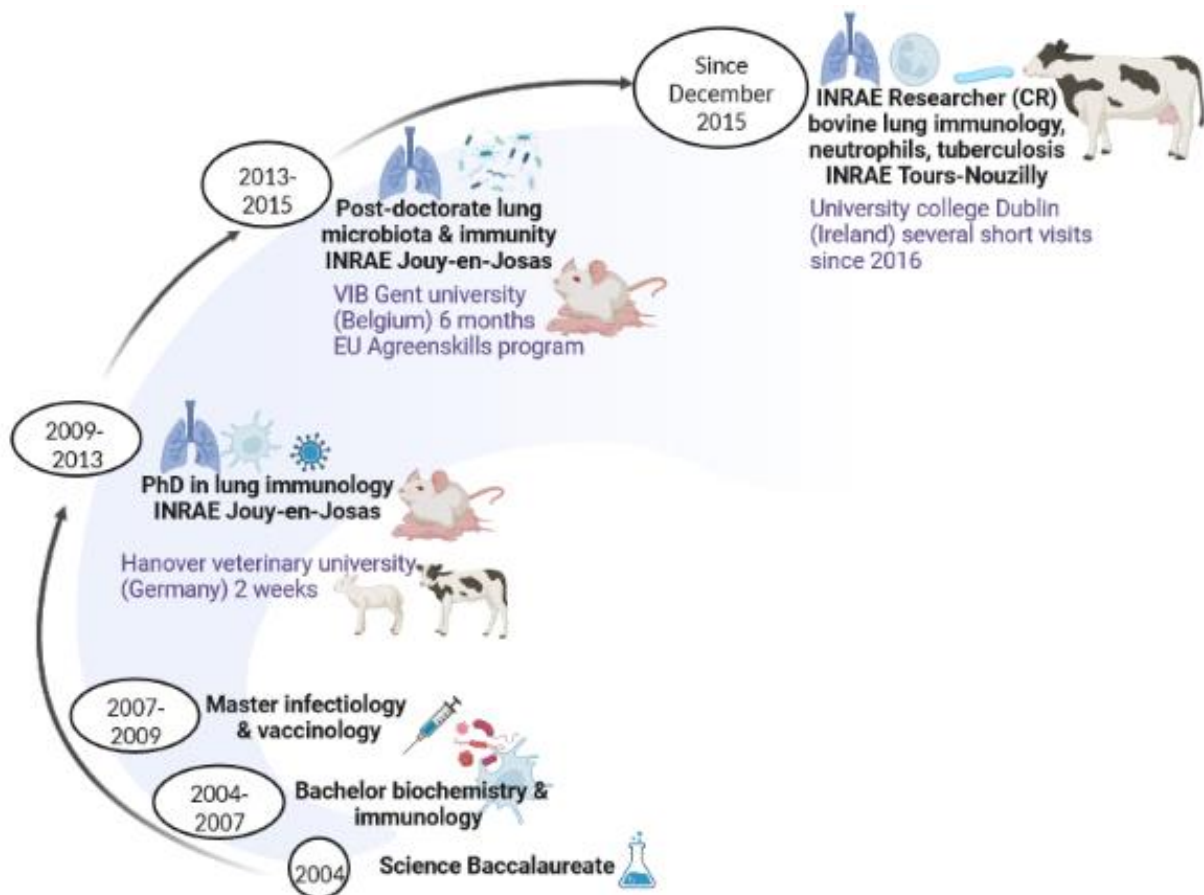
English / French

AM	Alveolar Macrophage
ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
ANR	Agence nationale de la recherche
ANR JCJC	Contrat ANR Jeune Chercheur Jeune Chercheuse
APC	Antigen Presenting Cell
BCG	Bacille de Calmette et Guérin
BRD	Bovine respiratory disease
bRSV	Bovine respiratory Syncytial Virus
bTB	Bovine tuberculosis
CHU	Centre Hospitalier Universitaire
CIFRE	Conventions industrielles de formation par la recherche
CJS	Contrat Jeune Scientifique
DC	Dendritic cell
FLI	Friedrich Loeffler Institute
HDM	House dust mite
hRSV	Human respiratory Syncytial Virus
PHC	Programme Hubert Curien
IBIR	Equipe Infections Bactériennes et Immunité des Ruminants
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukine
INRAE	Institut national de recherche pour l'agriculture, l'alimentation et l'environnement
LPS	Lipopolysaccharide
MDSC	Myeloid Derived Suppressor Cells
Mb	<i>Mycobacterium bovis</i>
MHC-II	Major histocompatibility complex class II
MLR	Mixed Lymphocyte Reaction
Mtb	<i>Mycobacterium tuberculosis</i>
PCA	Principal Component Analysis
PCLS	Precision Cut Lung Slices
PCR	Polymerase chain reaction
PMN	Polymorphonuclear
ROS	Reactive Oxygen Species
RSV	Respiratory syncytial virus
RT	Reverse transcription

RT-qPCR	Real-Time quantitative reverse transcription PCR
SCC	Somatic Cell Count (in milk)
SEM	Standard Error of the Mean
SPF	Specific Pathogen Free
TB	Tuberculosis / <a href="#">Tuberculose</a>
Th	T helper
TLR	Toll Like Receptor
TST	Tuberculin Skin Test
UCD	University College of Dublin
<a href="#">UMR</a>	<a href="#">Unité Mixte de Recherche</a>
<a href="#">VRS</a>	<a href="#">Virus Respiratoire Syncytial</a>
WHO	World Health Organization
WP	Workpackage

## 2. Introduction

This HDR manuscript is a presentation of my career trajectory with an overview of my research activities as a lung immunologist since my PhD.



My passion for science and immunology is the reason why I have decided to specialize in life-science research. My Master focused on infectiology and vaccinology, I undertook many courses on mucosal immunology and host/microbes' interactions, in many types of mucosal tissue including the respiratory tract.

I went deeper in the respiratory field during my PhD, under the supervision of Dr. S. Riffault (UMR0892 VIM, INRAE Jouy-en-Josas), where I investigated the immune factors responsible for neonatal sensitivity to Respiratory Syncytial Virus (RSV). I obtained a competitive PhD INRAE contract ("Young Scientist Contract") and for three and a half years, through experiments, bibliography, networking, and meetings, I was able to highly specialize in lung immunology. I studied the specificity of neonatal immunology in three different contexts: at steady-state, after infection with RSV and anti-RSV vaccination. I used a neonatal mouse model to decipher the immuno-pathological mechanisms and worked with livestock species, lambs and calves. I deciphered several immune deficiencies (type I interferon, dendritic cells) contributing to the greater sensitivity of newborns to RSV. I strengthened my skillset in immunology and gained expertise in transcriptomics (microarray) and lung explants (training in Hanover University, Germany). I learnt how to work in field condition (experimental farms for lambs, commercial



abattoirs for calves) and how to organize my work with complex experiments (surgery for preterm lambs for instance).

At the end of my PhD, I wanted to explore a new and promising area: the lung microbiota, which had recently been discovered. I wrote my own post-doctoral project to join the ProbiHote team (UMR1319 MICALIS, INRAE Jouy-en-Josas). I acquired the necessary expertise in microbiology to develop the subject on the role of microbiota on lung immunology under the supervision of Dr. M. Thomas. I chose this team for its expertise in physiology and gut microbiota and I brought to the laboratory my knowledge in lung immunology. To evaluate the impact of lung microbiota in the context of lung disorders, I chose to work on a model of induced asthma. I obtained an European mobility grant (Agreenskills fellowship) to spend 6 months in Ghent in Belgium, to work alongside Pr. B. Lambrecht and Pr. H. Hammad, where I conducted experiments using their House Dust Mite induced model of asthma in mice. During my post-doctorate, I isolated primo-colonizing lung bacteria in neonatal mice, and identified two strains with antagonist effects: one exacerbating asthmatic lung inflammation, another one with a protective effect. With this project, I showed that I was able to carry a research project from its conception to its completion. I adapted to new work environment, a state-of-the-art microbiology unit and a renowned immunology laboratory abroad. I diversified my expertise with bacteriology and the manipulation of germ-free mice. I applied to a Marie-Curie fellowship, obtained a grant from Paris-Saclay IDEX, and had several contacts with private companies and hospitals as my own research funding's.

In 2015, I underwent a competitive INRAE selection process and was successful in obtaining a permanent researcher position. I joined the UMR1282 ISP unit as a permanent researcher (Chargée de Recherche) to work on the physiopathology of bovine tuberculosis (bTB), under the mentorship of Dr. N. Winter. I implemented the work on the target species i.e cattle. My current research projects are oriented toward the understanding of innate immune factors in the lung which can contribute to the control of *Mycobacterium bovis* (Mb), the causative agent of bTB. I also have a strong interest for the response of bovine neutrophils, and recently characterized a subset of bovine neutrophils with regulatory properties. Since my recruitment, I have built a professional network, within France and also at the European level with Germany and Ireland. Through this, I have co-coordinated two Program Hubert Curien (PHC) exchange projects with the University College of Dublin (UCD), welcomed a post-doc and a PhD student for short stays and myself spent several short times at UCD. I developed an immunological and molecular toolbox for cattle, obtained several grants and conducted various research projects. This year, I obtained an ANR JCJC grant, to decipher the role of bovine neutrophil subsets in the physiopathology of Mb. With my interests and expertise in bovine neutrophils, I also contribute to research related to bovine mastitis, the other cattle disease studied in my team, and since 2019, I am the supervisor of a PhD Student, Marion Rambault (her PhD project is focusing on the role of bovine neutrophils in the context of mastitis).

### 3. Curriculum Vitae

#### Personal Information

Name: Remot-Brizion, Aude  
Nationality: French  
Date of birth: 8 February 1987  
Personal situation: married, two daughters, born in 2016 and 2019



#### Education

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01/10/2009 – 28/02/2013	PhD in Immunology (very honourable distinction) Paris Institute for Life, Food and Environmental Sciences (AgroParisTech). ABIES doctoral school, Paris, France
03/09/2007 – 30/06/2009	Master degree in Cellular and Molecular Infectiology and Vaccinology (Rank 1/41, Magna cum laude distinction) University of Sciences and Techniques, Tours, France
01/09/2004 – 29/06/2007	Bachelor degree in Biochemistry (major Immunology) University of Sciences and Techniques, Tours, France
2004	Sciences Baccalaureate

#### Current position

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Since 01/12/2015	Researcher (Chargée de Recherche classe normale, CRCN), INRAE département MICA Main project: “Bovine innate responses in the context of major bacterial infection”. Team Immunology and Bacterial Infections of Ruminants (IBIR), UMR1282 ISP unit, INRAE Centre Val de Loire, France
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#### Previous positions

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01/03/2013 – 31/12/2014	Researcher (Post-doctorate, INRAE Young Scientist Contract) Project: “Impact of the development of the lung microbiota on neonatal immunity”. Team Commensal and Probiotics-Host Interactions (ProbiHote), UMR1319 MICALIS unit, INRAE Jouy-en-Josas, France. Supervisor: Dr. Muriel Thomas
01/09/2013 – 31/03/2014	Agreenskills visiting scientist (6-month mobility during my post-

	doctorate, EU Agreenskills fellowship)
	Project: “Impact of the development of the lung microbiota on neonatal immunity”. Laboratory of immunoregulation and mucosal immunology (Inflammation research center, VIB), Ghent, Belgium.
	Supervisor: Pr. Hamida Hammad & Pr. Bart Lambrecht
01/10/2009 – 28/02/2013	PhD Student (INRA Young Scientist Contract)
	PhD Project: “Immune lung features related to the higher severity of respiratory syncytial virus infection in newborns”. Team Vaccination and Antiviral Immunity (V <sub>2</sub> l), UMR0892 VIM unit, INRAE, Jouy-en-Josas, France
	Supervisor: Dr. Sabine Riffault
05/01/2009 – 30/06/2009	Master2 Internship
	Project: “Evaluation of the vaccinal potential of a metabolically biotinylated adenovirus for ovine dendritic cell targeting and ligand screening”. Team Apicomplexa and mucosal immunity (AIM), ISP unit, INRAE Centre Val de Loire, France
	Supervisor: Dr. Fabrice Laurent
03/03/2008 – 30/04/2008	Master1 Internship
	Project: “ <i>In vitro</i> modulation of CD8+ T cells “educated” by human dendritic cells pre-treated with mycophenolic acid”. Dendritic Cells and Graft Laboratory, Bretonneau Hospital, Tours medical university, France
	Supervisors: Dr. Roxane Lemoine & Dr. Florence Velge-Roussel

## Teaching activities

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2017 to present	1 lecture per year on mycobacterial immunity (1h30) for Master2 students in Infectiology at Tours university (in English, lecture for both the national Master I <sup>2</sup> VB and the IDOH Erasmus Master students)
16 and 17/06/2022	Introduction to Science and Immunology for children (3 to 12 years old), kindergarten and elementary school, Semblancay.
11/2015	1 lecture on lung immunity for 3 <sup>rd</sup> year med students and project tutor for a group of 5 students, CHU Saint Antoine, Paris.
2014 and 2015	Teaching position in Cergy-Pontoise University, France (co-responsible of the Master teaching module on molecular immunology). 11 lecture courses and 3 practical courses, 26h in total.
04/02/2011 and 27/01/2012	Lectures on vaccination for engineer students in Agronomy, Paris, France. 2x1h.

## Institutional responsibilities

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04/12/2012	Representant of young INRAE scientists for a round table during a public audition organized by Office parlementaire d'évaluation des choix scientifiques et technologiques, Assemblée Nationale, Paris
01/09/2010 – 31/08/2012	Member of Doc'J association (treasurer), INRAE, Jouy-en-Josas, France (& organization of the annual PhD day)
01/09/2010 – 31/08/2011	Member of ABIES doctoral school scientific committee, Paris, France

### Memberships of scientific societies

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2011 to date	Member of the French Society for Immunology (Immunology Ambassador for kids in 2022)
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### 19 publications accepted in peer reviewed journal

Position	Number of publications	Journal and year of publication
1 <sup>st</sup>	7	INRAE Prod. Animales (2022, in press), Frontiers in Vet. Science (2021) Frontiers Immunol (2019), ISME J (2017), Eur. J Immunol (2016 et 2011), PloSONE (2012)
Last	2 + 1 under review	Frontiers Immunol (2021), Methods Mol Biol (2021), J.Dairy Science 2022 under review (preprint in BioRxiv)
2 <sup>nd</sup>	2	FASEB J (2016), PloSONE (2010)
Before last	1	Frontiers Physiol (2018)
other	7	FASEB J (2021), Journal of Immunology (2021), Vaccines (2021, 2020), Frontiers Immunol (2019), Nat Commun (2014), Vet Res (2011)
<a href="https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date">https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date</a> ORCID ID : <a href="https://orcid.org/0000-0001-9896-3216">0000-0001-9896-3216</a>		
1 Patent Co-inventor (45%), n°1650565, PCT/EP2017/051839		
3 DIRV - invention Disclosures (inventor at 30%, 20% and 15%)		

### Communications

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17 oral presentations (outside of INRAE), 7 as invited speaker, 10 selected from abstracts. 2 prizes for best presentation.

20 posters, 2 best poster prizes.

### Grant obtained

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ANR JCJC Neuro-bTB (Coordinator, 346k€, 2022-2025), Fédération de recherche en Infectiologie 'Mycovid' (co-coordinator, 10k€, 2022-2023), APIS Gene 'MASTICELLS' (WP leader, 424k€, 2019-2022),

PHC Ulysses 'NeuBoViD' (co-coordinator, mobility grant, 2021) and 'ONETB' (co-coordinator, mobility grant, 2018), Institut Carnot F2E 'BoNeutro' (coordinator, 70k€, 2018-2019); APIS Gene 'HealthyCalf' (WP leader, 579k€, 2017-2020), INRAE Young Scientists projects (15k€ in 2017 MICA; 10k€ in 2016 SA), Prématuration Idex Paris-Saclay (co-coordinator, 80k€, 2015), INRAE AlimH division support (3 months post-doc salary, 2014), EU Agreenskills fellowship (mobility grant, 2013), INRAE Young Scientist Contract (2009-2013).

## Mentoring

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PhD Student (co-supervision, since 11/2019), Master2 students (2019, 2018, 2010), Master1 students (2022, 2021, 2017), research engineer (2015), professional bachelor's student (2014, 2021), Bachelor student (2014, 2015)

## Scientific expertise

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**Review editor** for Frontiers: Microbial immunology & Veterinary Infectious Diseases (since 2017), occasional reviewer for other journals (related to veterinary sciences or immunology) ≈ 5 manuscripts per year

**DIM1Health grant evaluator** (2018, 2022)

Member of 3 **PhD committees** (Comité de Suivi Individuel, 2 PhD students defended their PhD in 2018 and 2019, the 3<sup>rd</sup> one started in 2021)

**Examiner** for 1 Science PhD (Examinatrice, 2019)

**Reviewer** for 1 Pharmacy thesis (Rapporteur, 2017)

**Jury** for Tours Master 1 and Master 2 students (since 2016)

## Scientific and technical skills

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**Project management:** bibliography, conception of protocols, realization and analysis of experiments, redaction of scientific articles, project research, coordination of research projects (Institut Carnot F2E, PHC, ANRJ/CJ)

**Immunology:** Flow cytometry, ELISA, MLR, phagocytosis and viability assay, histology (IF and IHC), confocal microscopy

**Microbiology:** virology (respiratory syncytial virus), bacteriology (isolation and characterization of strains, collection management, anaerobic strain manipulation in Freter Chamber, mycobacteria), work in BSL2 and BSL3.

**Cellular biology:** cell line culture, obtention and culture of *ex vivo* explants (Krumdieck Tissue Slicer), epithelial cells culture in Air-Liquid interface, isolation of primary cells (neutrophils, macrophages, dendritic cells, lymphocytes...).

**Molecular biology:** DNA or RNA extraction, RT, RT-qPCR (Syber or Taqman, eukaryote and prokaryote, medium throughput qPCR with the Fluidigm Biomark technology). Transcriptomic analysis (Genespring, Ingenuity Pathway Analysis, Real-time PCR softwares).

**Biochemistry:** Western Blot

**Animal experimentation:** FELASA category C certification in 2011 (French habilitation niveau 1 concepteur), murine models (work in level A2 biocontained facility, isolators with germ-free and gnotobiotic mice) and ruminants (sheep and cattle).

**Statistical analysis:** GraphPadPrism and R (RStudio)

## 4. Research activities

### A. Immunological signature of neonatal susceptibility to respiratory syncytial virus infection (PhD)

#### i. Context and state-of-the-art



Childhood, noticeably the neonatal period, is marked by a high susceptibility to infectious diseases. About 20% of all deaths in children under 5 years of age are due to acute lower respiratory infections (pneumonia, bronchiolitis and bronchitis) caused by bacteria (most commonly *Streptococcus pneumoniae* and *Haemophilus influenzae*) or viruses (most commonly respiratory syncytial virus (RSV)) (Nascimento-Carvalho et al., 2001)(Hall et al., 2009).

The orthopneumovirus RSV is widespread around the world, and virtually every child has been infected with this virus by the age of two (Nair et al., 2010). About 1/3 of human RSV (hRSV) infections spread to the lower respiratory tract, causing bronchiolitis and pneumonia. As a result of hRSV infections, between 1 and 3% of children in the developed world are hospitalized in pediatric intensive care units in the winter season, and >100,000 children each year die of hRSV infections in the developing world (Shi et al., 2017). hRSV is also an important trigger of asthma exacerbations, since half of children with mild to severe RSV bronchiolitis will develop recurrent wheezing or asthma (Sigurs et al., 2004).

Bovine RSV (bRSV), genetically and antigenically closely related to hRSV, induces an acute lower respiratory disease in calves that shares many clinical and virological features of infant RSV-bronchiolitis (Sacco et al., 2014). Calves acquire maternally derived serum antibodies at birth by drinking colostrum from their bRSV-immune mothers, and young calves are most susceptible to severe bRSV disease when passive immunity from maternally derived serum antibodies has decreased to moderate or low levels, usually at one month of age (Kimman et al., 1988). bRSV is estimated to cause 14 to 71% of bovine respiratory disease outbreaks (Ames, 1997; Sarmiento-Silva et al., 2012).

The higher susceptibility of neonates to infectious diseases is generally attributed to the “immaturity” of their immune system. The neonatal T helper (Th) responses have been well characterized in one-week old mice. C57BL/6 and BALB/c mice are Th1- or Th2-dominant, respectively. BALB/c neonates commonly demonstrate enhanced Th2 functions at all steps of the immune response and poor Th1 memory responses resulting in an overall Th2-dominance during early life (Zaghouani et al., 2009). This Th2 immune profile is an intrinsic feature of the CD4+ T cells within the secondary lymphoid organs. Indeed, when extracted from their neonatal environment and transferred into adult recipients, CD4+ T cells produce high levels of the type 2 cytokine IL-4 and are deficient for the production of the type 1 cytokine IFN- $\gamma$

(Adkins et al., 2002). Two mechanisms may contribute to the neonatal Th2 bias. First, the Th2 cytokine locus is hypomethylated during the neonatal period, thus allowing rapid high-level Th2 gene transcription (IL-4, IL-5 and IL-13) (Rose et al., 2007). Second, neonatal Th1 cells express high levels of interleukin 13 receptor  $\alpha 1$  (IL-13R $\alpha 1$ ), and are prone to apoptosis driven by IL-4, which is produced by activated Th2 cells (Lee et al., 2008). Furthermore, BALB/c neonates lack the splenic CD8 $\alpha$ +CD4 $\beta$ - dendritic cell (DC) subset that is required for IL-12-mediated downregulation of IL-13R $\alpha 1$ , allowing Th1 memory to arise (Lee et al., 2008). It is nevertheless possible to generate adult-like immune responses during the neonatal period, if the appropriate stimuli and cellular partners are gathered in the right settings (Ridge et al., 1996). For instance, CpG oligodeoxynucleotides added to protein or live vaccine like the Bacillus Calmette Guérin (BCG) can circumvent the neonatal Th2 polarization (Kovarik et al., 2001, 1999; Murray et al., 2006; Sanchez-Schmitz and Levy, 2011). Vaccination faces multiple challenges such as the identification of safe and effective adjuvants, innovative antigen discovery, and delivery strategies, all adapted to the targeted population (Lavelle and Ward, 2022). In the mid-1960's, a clinical trial of an inactivated RSV vaccine led to a 80% increase in hospitalizations of vaccinated infants naturally infected with RSV during winter, and two deaths (Kapikian et al., 1969). To date, there is no approved vaccine to prevent RSV infection but candidates are currently in clinical trials (Blanco et al., 2018).

Only 2–3% children, mostly before 6 months of age, experience an acute lower respiratory disease requiring hospitalization (Lambert et al., 2014). These variations in disease severity seem mostly related to polymorphisms in innate immunity genes, including many that are part of the interferon (IFN) system (Mejias et al., 2013). Moreover, infants with severe bronchiolitis have elevated IL-4/IFN- $\gamma$  ratio in nasal washes, which suggest that disease severity is associated with an excess of type 2 immune responses (Legg et al., 2003). However, the immunological pathways leading to Th2-driven acute bronchiolitis have not been fully elucidated (Busse et al., 2010; Lambert et al., 2014; Pérez-Yarza et al., 2007). To get better picture of immunity to RSV in infants and its long-term impact on airway disease, a neonatal mouse model of RSV infection was developed (Cormier et al., 2010; Culley et al., 2002). When BALB/c mice are infected with RSV before 1 week of age, they mount poor Th1 responses characterized by little antiviral CD8 T cells producing IFN- $\gamma$  (Tregoning et al., 2008). Upon second exposure, adult mice initially infected as neonates display greater Th2 responses associated with aggravated airway inflammation (eosinophilia and mucus hyperproduction) (Culley et al., 2002).

## ii. Scientific questions

Immune responses against respiratory viruses are well described in adults, but less in neonates. Little is known about how innate pulmonary immunity matures with age and the influencing factors. The understanding of neonatal lung immune responses is a priority to fight RSV which affect infants and calves. The questions set at the beginning of my PhD were:

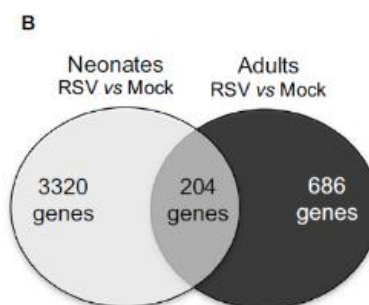
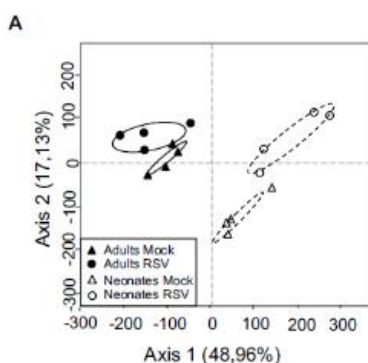
- Why do neonates display higher sensitivity to lower respiratory tract infections?
- How to vaccinate against RSV during the neonatal period without exacerbating lung pathology?

### iii. Main results

To address these questions, I worked in parallel with BALB/c mice (Th2 dominant and sensitive to hRSV) and with ruminants (bRSV). I organized my work in three complementary axes.

First, I explored the neonatal immunity in uninfected lungs. The main objective was to understand which factors, present at steady-state, induce different responses in newborns and adults. I was particularly interested in lung dendritic cells (DC) in BALB/c and C57BL/6 pups, and the orientation of Th lymphocytes. Higher expression of the transcriptional factor GATA-3 and rapid and sustained production of type 2 cytokines by lung explants after *in vitro* exposure to anti-CD3 was the hallmark of the neonatal period, suggestive of a Th2 bias. GATA-3-producing cells were identified as CD3+, CD4 and CD8 double-negative T lymphocytes, a subset found at a higher frequency in neonatal than adult lungs. The neonatal lungs contained fewer conventional DCs, with a ratio of CD103+ to CD11b+ DCs twice reduced and 4 times less plasmacytoid DCs in comparison to adult lungs. Yet, when stimulated *in vivo* with BCG, neonatal lung conventional DCs matured and primed adult naïve CD4+ T cells toward Th1 as efficiently as adult BCG-primed lung conventional DCs. Conversely, both adult and neonatal BCG-primed lung DCs induced a Th2 cytokine response from neonatal naïve lymph node T cells, suggesting that Th2 polarization is an intrinsic feature of neonatal T lymphocytes and that neonatal DCs are fully functional. This work was published in European Journal of Immunology in 2011 (Remot, Roux et al., 2011).

Second, I investigated the early events occurring in the lung upon RSV neonatal infection. I used a *no a priori* global approach, based on total lung transcriptomics (microarrays) comparing Mock and RSV infected neonate vs adult BALB/c mice. As observed by Principal



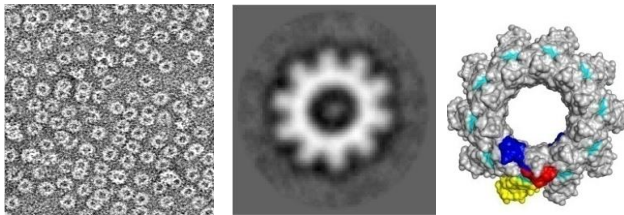
Component Analysis, differentially expressed genes depended on the age (Figure panel A). Only 204 differentially expressed genes were common between neonates and adults' lungs as depicted on the Venn Diagram (figure, panel B). I then

validated and extended these data by flow cytometry, ELISA, histology and q-RT-PCR, focusing on type I IFN and DC-related pathways that were severely impaired in neonates. Flt3 ligand is a hematopoietic growth factor that triggers expansion of cDCs and pDCs in human cord blood and strongly promotes IFN- $\alpha$  production by pDCs in response to viral exposure (Willems et al., 2009). I administered Flt3 ligand to mice during the neonatal period which led to a reduced lung pathology after RSV infection, without any effect on the Th2 immunity. These results



opened the door to reevaluate the dogma which linked the Th2 neonatal bias to severity of RSV pathology. This work was published in European Journal of Immunology in 2016 ((Remot et al., 2016).

Third, I worked to adapt a vaccine candidate against RSV (patented in the Molecular Immunology and Virology unit) to the neonatal period. Achieving safe and protective vaccination against RSV in infants and in calves has proven a challenging task. The candidate patent is the nanoparticle N<sup>SRS</sup> composed of 10 or 11 RSV nucleocapsid proteins which auto-assemble into a ring-shaped structure, as illustrated below (Tawar et al., 2009).

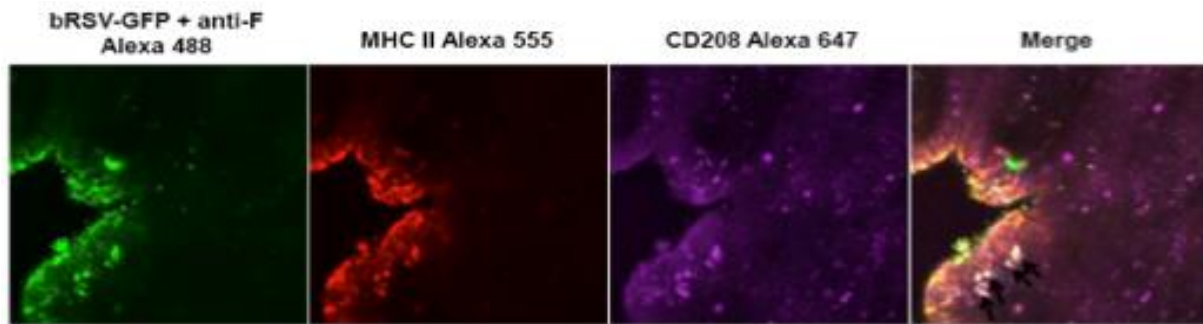


When administered intranasally, N<sup>SRS</sup> induce a Th1 immunity which protects adult BALB/c mice against RSV challenge, without any lung inflammation (Roux et al., 2008). My work in neonatal mice showed that the nature of adjuvants was a key element to

achieve protection against RSV without exacerbating lung inflammation. A single intranasal administration of N<sup>SRS</sup> with detoxified *E. coli* enterotoxin LT(R192G) to 5-7-day old neonates provided a significant reduction of the RSV load after challenge at five weeks of age. However, neonatal vaccination also generated an enhanced lung infiltration of neutrophils and eosinophils following the RSV challenge. Analysis of antibody subclasses and cytokines produced after RSV challenge or a booster of the vaccine suggested that neonatal vaccination induced a Th2 biased local immune memory. This Th2 bias and the eosinophilic reaction could be prevented by adding CpG to the vaccine formulation, which, however, did not prevent pulmonary inflammation and neutrophil infiltration upon viral challenge. This work was published in PLoS ONE in 2012 (Remot et al., 2012). Since that time, my PhD supervisor Dr. S. Riffault has conducted several vaccine studies in calves using N<sup>SRS</sup> and I had the pleasure to contribute to some experiments. I was recently associated to two publications on the subject (Riffault et al., 2020; Valarcher et al., 2021).

During my PhD, I also conducted experiments in two agronomical species: *ex vivo* work in calves and *in vitro* and *in vivo* work in lambs which are sensitive to bRSV, therefore a good pediatric model for lung deficiencies in preterm infants.

For the cattle model, I spent 2 weeks in Dr. G. Herrler laboratory in Hanover in Germany, to learn the lung explants technic using the Krumdieck Tissue Slicer (Precision Cut Lung Slices, PCLS), and the respiratory epithelial cells culture in Air-Liquid Interface (Goris et al., 2009). PCLS *ex vivo* infection by bRSV-GFP proved that in addition to bronchial epithelial cells (target cells), some cells close to the epithelium internalized the virus. Using confocal imaging, I showed that these cells were DCs; i.e. were directly infected or able to phagocytose infected cells (characterized as CD45, CD11c, MHC<sub>I</sub>, and CD208 positive, figure below).



For the sheep model, I carried out the experiments with a pediatrician who did her Master2 internship in the team. Together, we studied the expression of innate receptors: Toll like Receptor (TLR) and Protease activated receptor (PAR) by RT-qPCR from lymph nodes, trachea and lung biopsies. The expression of some receptors, TLR7 especially, was strongly down regulated in preterm lambs and neonatal lambs in comparison to adult sheeps. I also studied the sheep lung DCs, and found that the CD11c+ MHC<sub>II</sub>+ CADM1+ cDC subset (equivalent to murine CD103+ conventional DC) was under represented in young animals, and increased with age. This work confirmed our results obtained in the mouse model, and established the neonatal default in one conventional DC subset in two different species. Such neonatal deficit most certainly influences lung immune response during RSV infection. Finally, I participated in a lamb's vaccination study with N<sup>SRS</sup> with the objective to compare the intranasal and intramuscular route of administration, and to test a new adjuvant (unpublished work). I really enjoyed working with livestock species and the great collaborations with INRAE colleagues. Finding and adapting tools and protocols for veterinary species was challenging but very stimulating and rewarding on the personal point of view.

#### iv. Related publications

My PhD work was valorized by the publication of 3 research articles in which I am 1<sup>st</sup> author, presented here after in the HDR manuscript (and accessible via PubMed: PMID:21770043, PMID:22655066 and PMID:26681580).

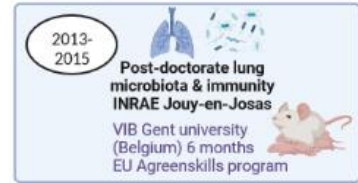
My contribution to the first article was: experiments presented in figures 2, 3 and 4 and the redaction of some sections of the article (preparation of figures 2, 3 and 4; redaction of materials/methods, legends of figures 2, 3, 4; and part of the results and discussion).

Regarding the second article, I performed all experiments presented, analyzed the data, prepared the figures, wrote the materials/methods and legends of figures, and reviewed the manuscript.

For the 3<sup>rd</sup> publication, I performed all experiments presented, analyzed the data, prepared the figures, and wrote the entire manuscript.

I was also associated to a publication in Nature Communications (5<sup>th</sup> author, (Rameix-Welti et al., 2014) PMID: 25277263). For this work I validated the visualization of a Luciferase-encoding hRSV in adult BALB/c mice by bioluminescent imaging, bioluminescence being detected in the snout and lungs of infected mice after nasal inoculation proving viral replication.

## B. Influence of lung microbiota on neonatal immunity (Post-doctorate)



### i. Context and state-of-the-art

While the healthy lung had long been considered sterile, this concept and the description of a commensal lung microbiota emerged in 2010 (during my PhD). As well-known for the gut, it is now admitted that the pattern of bacterial colonization in the respiratory tract influences lung health. Indeed, the bacterial colonization process in the intestine during the neonatal period is critical for the good development of mucosa and the balance of the immune system. The impact of the lung microbiota on the development neonatal lung immunity is still a poorly explored area. Yet, this may strongly impact the management of respiratory health of newborns. At the end of my PhD, I proposed a post-doctorate project to decipher how lung bacteria shape the innate and acquired respiratory immunity. For this, I proposed to combine *ex vivo* and *in vivo* strategies in mice. I worked with Dr. M. Thomas and Dr P. Langella, in the ProbiHote team in the MICALIS unit, at INRAE Jouy-en-Josas who are internationally recognized experts on the microbiota, and obtained an Agreeskills fellowship to spend six months in Pr. B. Lambrecht and Pr. H. Hammad laboratory, who are internationally recognized experts in lung immunology and asthma.

Allergic asthma is a worldwide health issue with rising prevalence and morbidity in developed countries. Variable airway obstruction, characteristic of asthma, is caused by chronic eosinophilic airway inflammation, mucus overproduction, airway wall remodeling and bronchial hyperreactivity. In allergic asthma, allergen-specific Th2 lymphocytes cause inflammation and control synthesis of allergen specific IgE, a hallmark of allergic sensitization (Hammad and Lambrecht, 2021). Pr. B Lambrecht and H. Hammad defined a key role for lung DCs and epithelial cells in bridging innate and adaptive Th2 immunity in asthma. All key features of Th2-driven asthma can be prevented by conditional depletion of DCs, identifying these cells as major actors in allergic sensitization to inhaled allergens (Hammad et al., 2010; Plantinga et al., 2013). Alternatively, DCs and epithelial cells also induce tolerance to harmless antigens (Juncadella et al., 2013). The cause of the epidemic of asthma in the Western world is unclear, but its increased incidence has been correlated with changes in environment and gut microbiota. First, the “hygiene hypothesis” proposes that a lack of early microbial stimulation in developed countries results in an aberrant immune response to innocuous antigens later in life (Wills-Karp et al., 2001). The “microbiota hypothesis” claims that the gut microbiota dysbiosis, due to antibiotic use and dietary changes, disrupts the mechanisms of mucosal immunologic tolerance (Noverr and Huffnagle, 2005). Epidemiological and clinical data (Hoskin-Parr et al., 2013), as well as experimental data obtained in mouse models (Noverr et al., 2004; Olszak et al., 2012), support this last hypothesis. It is well described in the gut that bacterial colonization during the first months of life is critical for the development and the balance of the immune system: it promotes the maturation of the epithelial barrier, the

set-up of immune tolerance, and improves stimulation of the immune system that helps preventing and/or resolving infectious diseases (Al Bander et al., 2020; Chen et al., 2021).

Back in 2013, occurrence of similar phenomenon of microbial education of the mucosal microenvironment in the lung was not established. Historically, healthy lungs were considered sterile, but the description of a commensal lung microbiota emerged in 2010 and revised this dogma. A bacterial community, with *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacteria*, and *Veillonella*, was described in the healthy human lung (reviewed in (Beck et al., 2012). However, the composition and the role of the lung microbiota remains largely unknown. Gollwitzer et al. demonstrated that microbial signals in the lung of neonatal mice improve the immune tolerance to allergens (Gollwitzer et al., 2014). This study highlighted the potential of an inter-disciplinary project (immunology, microbiology and physiology) in the field of respiratory health, and the importance of the neonatal period. The exploration of microbiota influence on lung immunity is still a new and promising area which impacts the prevention and management of pulmonary diseases like asthma.

## ii. Rationale and hypothesis

Since the gut microbiota clearly influences homeostasis and immune responses of the host, I hypothesized that lung microbiota also played a key role in educating the lung immune system. I believed that pulmonary microbiota could influence the outcome of the immune response to inhaled allergens and ultimately modulate the development of diseases like asthma.

## iii. Aim and research objectives

I aimed to investigate the impact of the lung microbiota composition on lung immunity and the potential protective/triggering effects of some lung bacteria on the development of asthma. I addressed the followed three complementary research objectives in order to:

- Describe and isolate the primo-colonizing bacteria arriving early in the lung of newborn conventional mice, using both molecular approaches and *in vitro* cultures.
- Characterize the lung colonizing microbiota in naïve and House Dust Mite (HDM) exposed animals and to study their immune-polarizing capacities
- Analyze the effects of identified bacteria on lung immune response to allergens

Animals used for this project were C57Bl/6 mice, commonly used to study respiratory diseases like asthma. I used conventional, gnotobiotic (inoculated with primo-colonizing bacteria) or germ-free newborn mice.

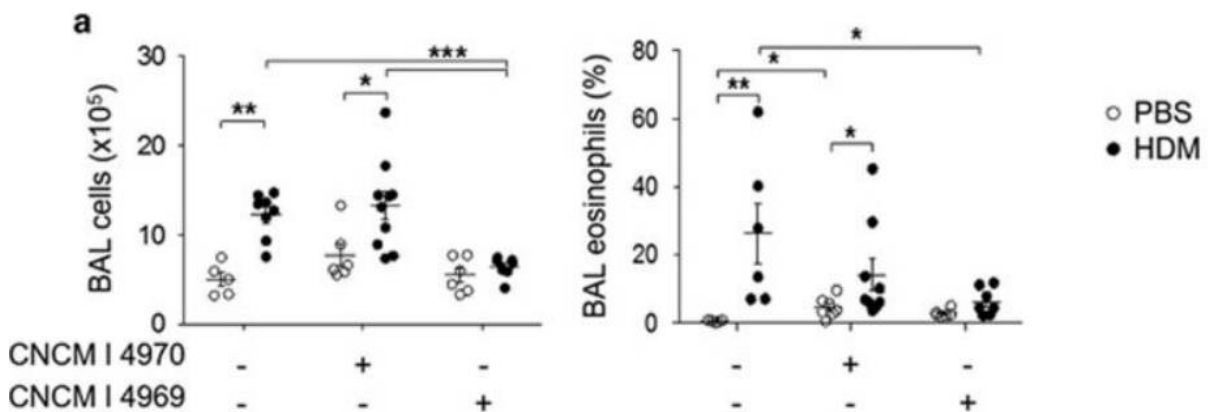
## iv. Main results

I set up a protocol to isolate and cultivate lung bacteria, both in aerobic and anaerobic conditions (Freter Chamber). I identified some of the cultivable primo-colonizing bacteria of

the lung during the neonatal period in naïve mice and put 26 strains in collection in the INRAE MICALIS unit.

I characterized these bacteria by screening their immunomodulating potential *ex vivo*: Precisely cut lung slices (PCLS) from germ-free (GF) neonatal or adult mice (considered as sterile naïve “tissues”) were co-cultured with the different bacteria. At different time points of incubation, I analyzed the cytokines released in the supernatants (ELISA), the lung cell proliferation or apoptosis (Flow cytometry, Immuno fluorescence and Westen Blot), and the mucus secretion (immunohistology). Four strains of particular interest were deposited at the Pasteur national collection (La Collection Nationale de Cultures de Microorganismes, CNCM).

Then, I validated the polarizing capacities of the 2 most promising bacterial strains (CNCM I 4969 and CNCM I 4870) by injecting them intranasally into GF animals, which were further subjected to the intranasal administration of HDM. I analyzed whether bacteria had the capacity to influence Th2 sensitization by analyzing the features of asthma (presence of eosinophilic airway inflammation, IgE, Th2 cytokines, mucus production) developed in these animals. CNCM I 4969 decreases some type 2 immune features whereas CNCM I 4970 worsens it, as illustrated by the eosinophilia in BAL below.



I established a cause-effect relationship between lung microbiota / lung immunity and asthma susceptibility. Part of my work was patented before publication.

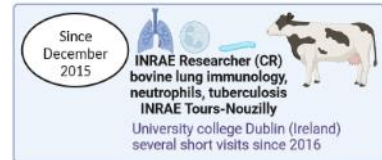
The elucidation of the microbiota contribution to severity or protection against asthma may lead to new treatments for patients, improving both health and welfare and possibly reducing economic costs of this invalidating disease. The use of pro- and prebiotics could reduce disease's risk. The identification of lung bacteria with immunomodulating properties against asthma during my post-doctorate led to spin-off activity and setting up of research contracts with biotech companies in and outside Europe. INRAE signed a contract with LALLEMAND company and a CIFRE PhD fellowship. One bacterial strain was also sent to a team in Toulouse for a PhD project on microbiota and tuberculosis (Bernard-Raichon et al., 2021). My former PhD team is now working with the ProbiHote team to decipher the role of lung microbiota for the control of RSV lung inflammation. They keep me in the loop of their project and results.

#### v. Related publications

My postdoctoral work was published in the ISME Journal in 2017 (Remot et al., 2017). I performed all the experiments presented in the article (both in France and Belgium), and wrote the entire manuscript. A patent was published before the article, I was co-inventor at 45%. In collaboration with Elliot Mathieu, a young research assistant whom I trained before leaving the team, I also wrote a review on lung microbiota, its relation to the lung physiology and the gut-lung axis (Mathieu et al., 2018). These two publications are presented here after and available online (PMID: 28045458 and PMID: 30246806).

I also participated in a study focusing on iron and highlighting new aspects of the crosstalk between the gut microbiota and the intestinal epithelium. The publication for which I am 2<sup>nd</sup> author (PMID: 26370847) was the first one to demonstrate that gut microbes induce a specific iron-related protein signature. I performed some experiments, prepared figure n°5, reviewed the manuscript and handled the revision.

### C. Bovine innate responses in the context of major bacterial infections (INRAE researcher)



After a competition on a permanent position that was opened in the Infectiology and Public Health unit at INRAE Centre Val de Loire, I arrived in the unit in December 2015, as a permanent researcher to study the physiopathology of bovine tuberculosis (bTB). In 2018, the mycobacteria team merged with another team studying bovine mastitis. Even though my research projects are mainly focused on bTB, I actively participate in mastitis related projects through the neutrophil angle.

#### i. Aims and research objectives

As a bovine immunologist working at INRAE, my ambitions are to:

- Develop immunological and molecular tools for cattle
- Understand the innate factors which contribute to Mb control in the bovine lung
- Identify correlates of protection against Mb infection

Since 2018, I have had a strong interest for neutrophils. The key role of neutrophils in human TB is established. Dual roles of inflammatory neutrophils and suppressive populations of granulocytic myeloid derived suppressor cells (MDSCs) - that are phenotypically close to neutrophils - on the fate of TB disease have recently been described in human TB patients and mouse models. The information on the role of bovine neutrophils during bTB is scarce. For the neutrophils related projects that I am conducting, I aim to:

- Better characterize neutrophils in cattle
- Decipher the role of neutrophil subsets in the pathogenesis of bTB

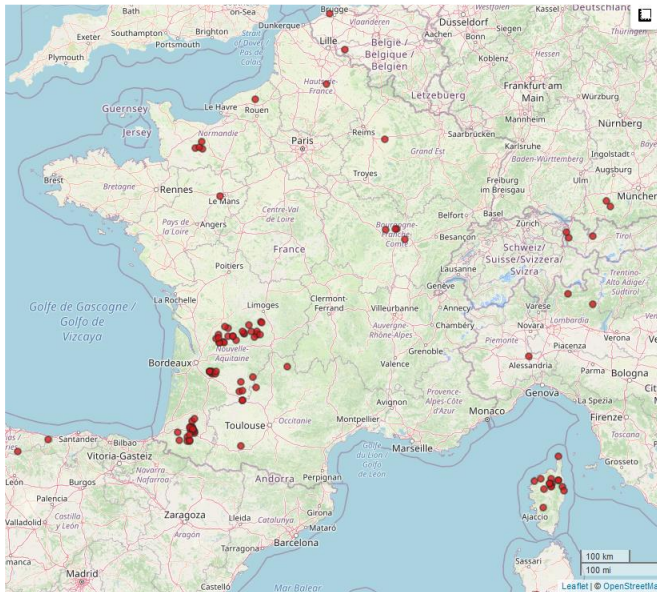
Neutrophils are key for the control of mastitis, and in the IBIR team we believe that improving the knowledge about these major players will lead to refinement of diagnostic or cattle genomic selection program. For the mastitis projects, I ambition to:

- Decipher the role of neutrophil subsets in the context of clinical and subclinical mastitis

#### ii. Bovine tuberculosis: context and state of the art



**bTB** remains one of the most challenging infections to control in cattle. It is caused by bacteria belonging to a complex of genetically similar lineages known as the *Mycobacterium tuberculosis* complex (MTBC). The canonical animal-adapted species, *Mycobacterium bovis* (Mb), is the main causative agent of bTB and is a multi-host pathogen capable of infecting wild-life reservoirs and humans (Olea-Popelka et al., 2017). Because of its zoonotic nature, the disease is under strict regulation in the European Union and the developed world and is associated with mandatory culling of affected animals. France is an officially bTB free country due to bTB surveillance programs costing around 20 million €/year (first item of expenditure



for the French Ministry of Agriculture). However, each year, one hundred Mb-infection foci are identified in France. The map on the left illustrates the bTB outbreaks reported in France between June 2021 and June 2022, (data from <https://shiny-public.anses.fr/shiny-vsi/>).

Eradication of bTB is difficult to achieve, because diagnostics lack specificity and sensitivity. Transcriptomic studies help refining and validating gene expression biosignatures (MacHugh et al., 2009). In developing countries where bTB surveillance programs are not always

implemented, bTB is an underestimated zoonosis and WHO has called for action to better tackle and if possible eradicate this disease (Olea-Popelka et al., 2017). Thus, bTB is identified as a very high priority disease by the EU and the STAR-IDAZ International Research Consortium on Animal Health (<https://www.star-idaz.net/priority-topic/bovine-tuberculosis-btb/>).

**The poor understanding of physiopathology and the lack of correlates of protection in the target animal, i.e. the bovine, is a main bTB knowledge gap** that must be covered to better tackle the disease. The physiopathology of human TB caused by *Mycobacterium tuberculosis* (Mtb) is better characterized and can be recapitulated as a continuum of host inflammatory response (Lyadova, 2017; Mayer-Barber and Barber, 2015). On one end of the spectrum, some individuals resist Mtb infection despite persistent and intense exposure. These persons do not exhibit adaptive immune priming as measured by tuberculin skin test (TST) and IFN- $\gamma$  release assay responses. This highlights the primordial contribution of the innate immune system to destroy bacilli. On the other end of the spectrum, active TB patients display exacerbated inflammation that destroys the lungs and leads to Mtb spread. In the middle of the spectrum, latency represents an equilibrium of T-cell immunity as measured by TST and IFN- $\gamma$  release assay, with no clinical signs: Mtb is contained inside key cellular structures called granuloma. Granulomas are shaped by the structured aggregation of immune cells in cascade and are classically composed, at their mature stage, by foamy and epithelioid macrophages, scattered



multinucleated Langhans giant cells and neutrophils surrounded by a ring of lymphocytes coated by a collagen dense fibrotic capsule (Ramakrishnan, 2012). One critical step in TB control relies on the granuloma encapsulation process, essential for constraining growth and spreading of mycobacteria. In mice, lesions or granulomas without proper encapsulation lead to increased mortality (Bouté et al., 2017; Kramnik et al., 1998).

**Alveolar macrophages (AMs) are Mtb or Mb first target cells.** Properly activated, AMs can destroy phagocytosed mycobacteria, notably via the secretion of reactive oxygen species (ROS) and reactive nitrogen species (RNI) (Cassidy and Martineau, 2014). However, some bacilli are able to escape killing, reprogram macrophages (especially their metabolism) and survive inside them, turning them into re-infection or dissemination foci (Hackett et al., 2020; Laval et al., 2021; Pieters, 2008). These chronic inflammatory areas evolve into granulomas to constrain bacilli. In humans, overwhelming inflammation that takes place in the lung, due to AM inability to eliminate bacilli, results in active TB. The role of bovine macrophages to control Mb was mainly studied with monocytes-derived macrophages (Castillo-Velázquez et al., 2013; Qureshi et al., 1996). Some recent studies with bovine AMs demonstrated their key role to initiate the inflammatory responses after *in vitro* infection and the hijacking of their intracellular machinery by Mb for its own survival (Nalpas et al., 2015; Queval et al., 2021).

The other major players are **neutrophils, key cells regulating the different stages of the TB disease spectrum.** Individuals in contact with active TB patients are less likely to be infected with Mtb if their peripheral blood neutrophil counts are elevated (Martineau et al., 2007). In resistant mouse models, neutrophils come in two different waves after Mtb infection: before and after the onset of adaptive immunity (Appelberg and Silva, 1989; Lombard et al., 2016). While the first wave phagocytoses mycobacteria *in situ* in the lung; the second T-cell dependent wave is seldom associated with bacilli. In response to Mtb, T-cell dependent neutrophils do not control Mtb growth but rather establish close contacts with T-cells (Lombard et al., 2016) helping to form the structured mature granuloma (Seiler et al., 2003; Yang et al., 2012). Therefore, **neutrophils** are key to **bacilli containment** either because they **directly kill bacilli** or because they **help formation of the beneficial granuloma**.

However, neutrophils are a double-edged sword because, when massively recruited, they are directly responsible for tissue necrosis, destruction, and infection dissemination (Dallenga et al., 2017). During active TB, they are the most represented cell type in sputum (Eum et al., 2010) and drive a type I interferon-inducible transcriptional signature in blood cells (Berry et al., 2010). A common feature to all TB susceptible animals that develop hypoxic necrotizing granulomas is the abundance of neutrophils (Kramnik et al., 1998; Mattila et al., 2015; Turner et al., 2003). Necrosis is driven by neutrophil-derived ROS and is required for Mtb growth after uptake of infected neutrophils by human macrophages (Dallenga et al., 2017). In C3HeB/FeJ mice, neutrophils dying by **necrosis or NETosis** rather than apoptosis drive the caseous necrosis and liquefaction process (Marzo et al., 2014). **The interactions with Mtb, that control the fate of neutrophils, drive disease physiopathology.**

Neutrophils have long been defined as a homogenous population based on their typical polylobed nucleus and a minimal set of markers. The picture has become more complex with the **description of suppressive neutrophils** and close relatives like Myeloid Derived Suppressor Cell (MDSCs), which largely share markers with neutrophils. MDSCs are an immature and heterogeneous population present at homeostasis and accumulating in pathological situations. MDSCs suppress T cell responses, via different mechanisms, including production of ROS, nitric oxide (NO) or arginase 1 (Gabrilovich and Nagaraj, 2009). **Granulocytic MDSCs** display the same morphology and phenotype as *bona fide* neutrophils from which they can robustly be distinguished only based on their suppressive function [20]. Expansion of MDSCs is observed in blood of active TB patients and healthy recently exposed contacts (Bronte et al., 2016; du Plessis et al., 2013). This correlates with enhanced L-arginine catabolism and NO production in plasma from active TB patients (El Daker et al., 2015; Yang et al., 2014). In resistant (C57BL/6) or susceptible (129S2) mouse strains, MDSCs are identified in the lung parenchyma during the course of Mtb infection where they suppress T cells (Knaul et al., 2014). They also vigorously ingest Mtb. In susceptible mice, MDSCs accumulate in higher numbers and phagocytose more bacilli as compared to resistant mice. Therefore, in susceptible mice, MDSCs could represent a **niche for Mtb replication**, helping the pathogen to **escape the immune system** (Knaul et al., 2014). Interestingly, Anca Dorhoi et al., demonstrated that **MDSCs support Mtb replication** within *in vitro* granuloma, and alters its structure (Agrawal et al., 2018). MDSCs are also associated to TB progression and lethality (Tsiganov et al., 2014). These findings emphasize the **important and dual contribution of neutrophils and phenotypically related Gr MDSCs to TB physiopathology**.

There is **little information on the role of bovine neutrophils during Mb infection**. As in mice, sequential study revealed the prominent neutrophil component of the host response to Mb in early-stage lesions (Cassidy, 2006; Cassidy et al., 1998). Wang et al. observed a neutrophil gene signature in Mb infected cattle (Wang et al., 2013a) but *in vitro*, depending on the cytokine environment, neutrophils seemed unable to eliminate the bacteria (Wang et al., 2013b). Another study suggested a deleterious role of neutrophils, possibly through strong IL-17 production, which contributed to the pro-inflammatory response in lung lesions, and the production of arginase decreasing Mb killing (Shu et al., 2014). Interestingly, Mb exploits the type I IFN signaling pathway for its own survival in bovine macrophages (Chunfa et al., 2017; Ji et al., 2019; Wang et al., 2019). Whether, as demonstrated in human active TB patients (Berry et al., 2010), neutrophils contribute to this type I IFN signature during Mb infection in cattle remains unknown.

There is a **heterogeneity in the host's immune response to Mb infection** and this directly influences the risk of transmission from infected cattle. In the best-case scenario, the pathogen is cleared by the host innate immune system. Natural resistance to TB and bTB are linked to the host genetics under the combination of a large number of loci (Ameni et al., 2007; Raphaka et al., 2017; Wilkinson et al., 2017). Genetic selection of cattle would greatly

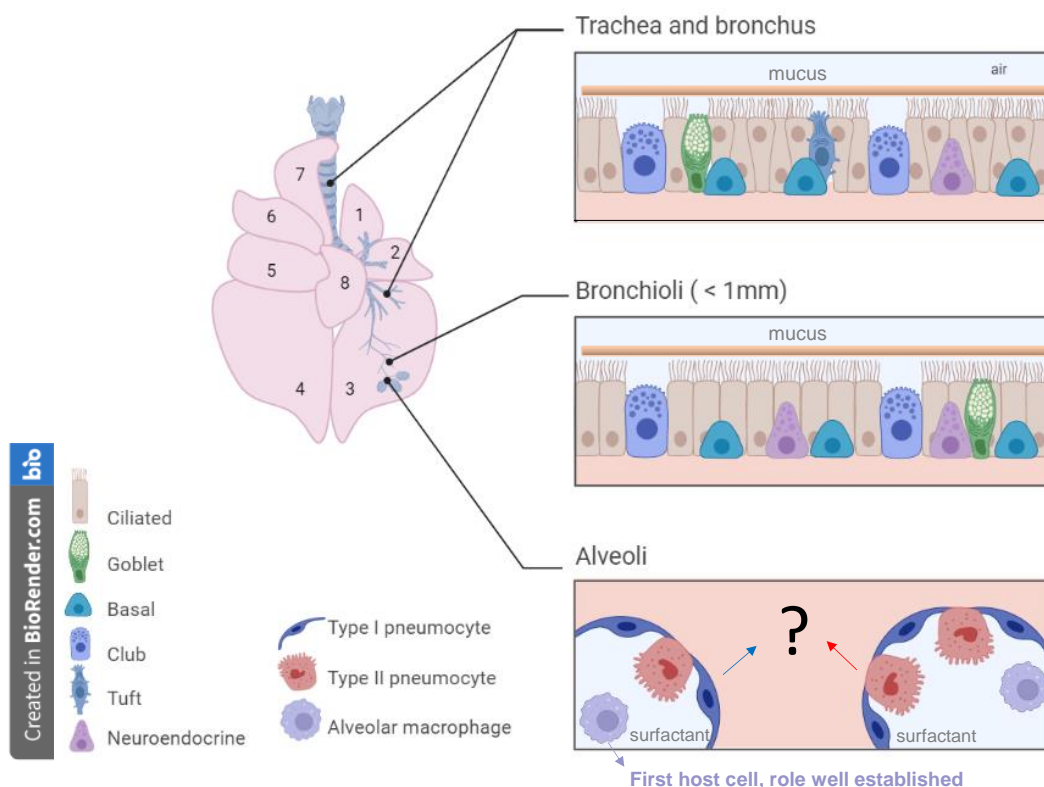
complement bTB management and surveillance programs to control and ultimately eradicate the disease, but **biomarkers to evaluate resistance or susceptibility** of cattle to Mb infection are critically **missing** along with the establishment of correlates of protection.

There is a rich history of codiscovery in the human and bovine TB field (Waters et al., 2011) and I believe in opportunities to learn from cattle experiments, as TB/bTB are a good example of the One Health concept.

### iii. Deciphering the early lung responses to mycobacteria

When I arrived in my unit, I implemented the model of lung explants called Precision cut lung slices (PCLS) which I learnt during my visit in Hanover during my PhD.

I wanted to investigate the bovine innate response following Mb infection in a preserved lung environment to allow the resident lung cells to interact with bacilli and crosstalk. PCLS are an experimental model in which resident lung cell types are preserved and remain alive for at least one week (Goris et al., 2009). The tissue architecture and the interactions between the different cells are maintained, as illustrated by the two figures below. The first one is a scheme to explain lung architecture, the second is a panel of PCLS pictures taken under a light microscope.



Bovine lung structure and cell composition, from the review Remot et al, 2022: Bovine lungs are composed of heights lobes. Depending on the compartment, different epithelial cells types are found. In bronchus or bronchiole, epithelia are composed by ciliated cells, goblet cells which produced mucus, basal cells, club cells, neuroendocrine cells and few tuft cells. Further, alveoli composed air sacs where

gas exchange occur. Alveoli are formed by long type I pneumocytes, and 1 or 2 type II pneumocytes which secrete surfactant and serve as progenitor for type I pneumocytes. Inside alveoli, resident AMs patrol the lung to ensure homeostasis. This figure is from a review in *Productions Animales* (Remot et al., *in press*).

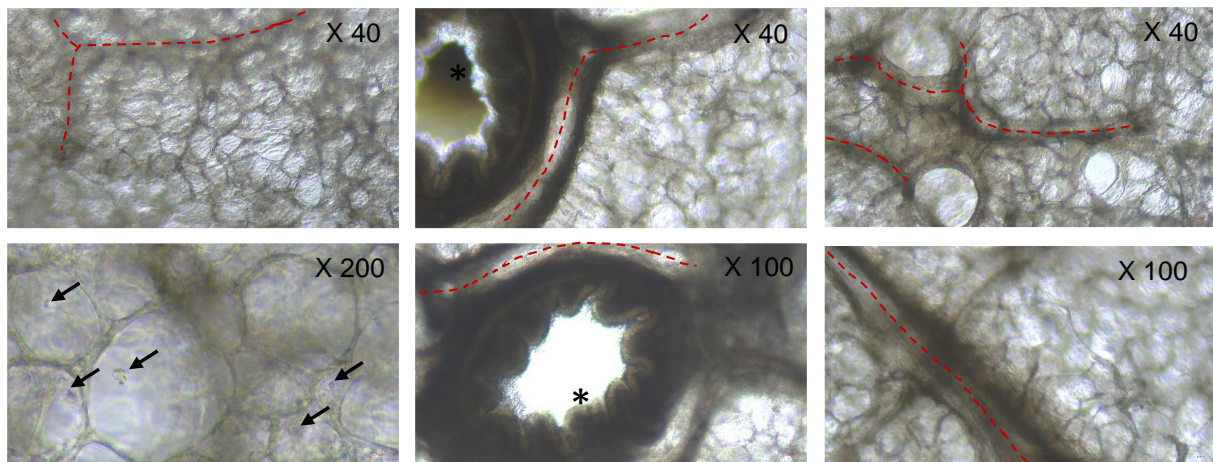


Figure S4 from the article Remot et al., 2021: Structure of bovine PCLS under light microscope. PCLS were observed under a light microscope (enlargement x40 to x200). PCLS contain numerous alveoli and between one to three bronchioles, with thick and wavy epithelium that can be easily recognized (black asterisk). Thin blood vessels (red dotted lines) were located next to bronchioles and diffused between alveoli. No blood cells remained inside the endothelium (cows were bled out at the abattoir). Alveolar macrophages can be seen inside alveoli (black arrows).

PCLS have already been validated for the study of various respiratory pathogens (Carranza-Rosales et al., 2017; Goris et al., 2009; Marquant et al., 2021). In chicken PCLS, mononuclear cells are highly motile and actively phagocytic (Bryson et al., 2020). This model is well designed to study complex interactions taking place early after the host-pathogen encounter. During Mb infection in cattle, important differences in production of key proinflammatory cytokines such as IFN $\gamma$  or TNF $\alpha$  by peripheral blood mononuclear cells are observed depending on the clinical status of the animal. Interestingly, such differences are observed at early time points (Thacker et al., 2020) indicating that the innate phase of the host response is key to the establishment of the pathological outcome of the infection.

Therefore, the PCLS model is ideally suited to investigate early host-pathogen interactions in the bovine lung during Mb infection, and may help to find clues on the role of the innate response on the outcome of the infection. This model, that fully mimics the early environment of the bacillus entering the lung, in contrast to monocyte-derived MPs, may also help understanding the molecular basis of mycobacterial host preference (Malone et al., 2018). To this end, I decided to compare Mtb and Mtb mycobacterial strains, in collaboration with Pr. S. Gordon from UCD (PHC project ONE-TB, co-coordinated by Pr. Gordon and myself). Since the host genetic background also has profound impact on the outcome of bTB disease (Allen, 2017), I decided to compare PCLS from two prevalent beef breeds in France, Charolaise and Blonde d'Aquitaine, and conducted a thorough characterization of the lung responses to Mb and Mtb during *ex vivo* infection. We first demonstrated that the French strain Mb3601 was

internalized by AMs in the preserved lung structure from PCLS, and AMs were recruited to alveoli containing mycobacteria (figure below, Remot et al., 2021).

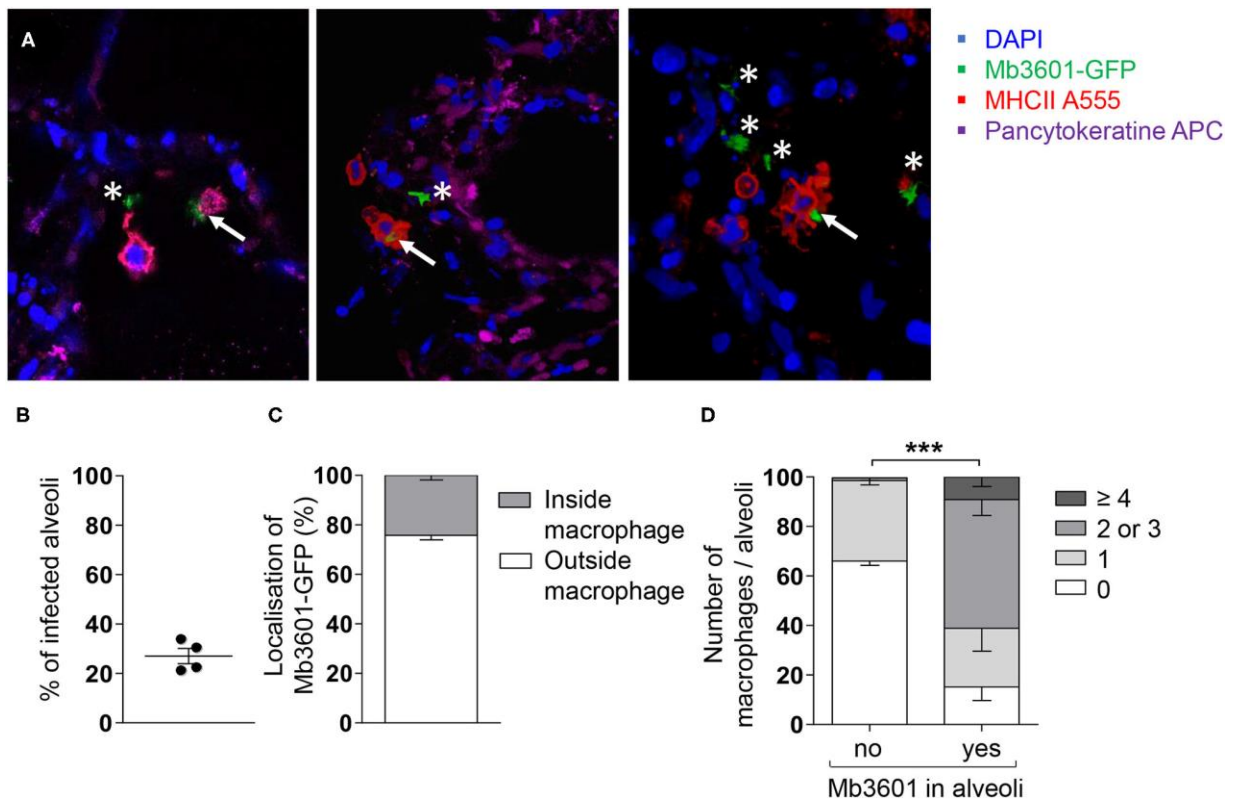


Figure 2 from the article Remot et al., 2021: Mb3601 is internalized by AMs in the preserved lung structure from PCLS and infected alveoli contain higher numbers of AMPs as compared to non-infected alveoli. PCLS were infected with  $10^5$  CFUs of the green fluorescent Mb3601-GFP recombinant strain and fixed 2 days later. After labelling with anti-pancytokeratine (APC, magenta) and anti-MHCII antibodies (Alexa 555, red), PCLS were mounted with Fluoromount-G™ Mounting Medium containing DAPI (blue) and analyzed under a Leica confocal microscope (A); 3D images were analyzed with Leica LAS software. Z-stack imaging was performed at x63 enlargement (10-15 $\mu$ m of thickness, step size of 0.5-1 $\mu$ m). White asterisks indicate extracellular bacilli and white arrows indicate bacilli inside MHC-II<sup>pos</sup> AMs. (B) Graph represents the percentage of infected alveoli per PCLS among the 55 to 80 alveoli that were observed under the microscope (n=4 PCLS from two different Blonde d'Aquitaine cattle) (C) Stack histogram of the mean percentage +/- SEM of intra or extracellular bacilli among a minimum of 15 infected alveoli that were observed (N=4 PCLS) (D) The number of MHC-II<sup>pos</sup> AMPs per alveoli was counted in infected or non-infected alveoli. The data presented as % are the mean +/- SEM of n=4 PCLS from two different Blonde d'Aquitaine cattle. Between 55 and 80 alveoli were observed to obtain these data (two-way ANOVA, \*\*\* p<0.001)

PCLS allowed us to decipher important differences in the transcriptomic and cytokine profile during the innate response to infection, depending both on the breed, i.e. between Blonde d'Aquitaine and Charolaise cows (with higher level of responses in Blonde d'Aquitaine), and on the mycobacterial species, i.e. between Mtb and Mb. Interestingly, type I IFN was induced by Mb but not Mtb in bovine PCLS as illustrated by the figure below.



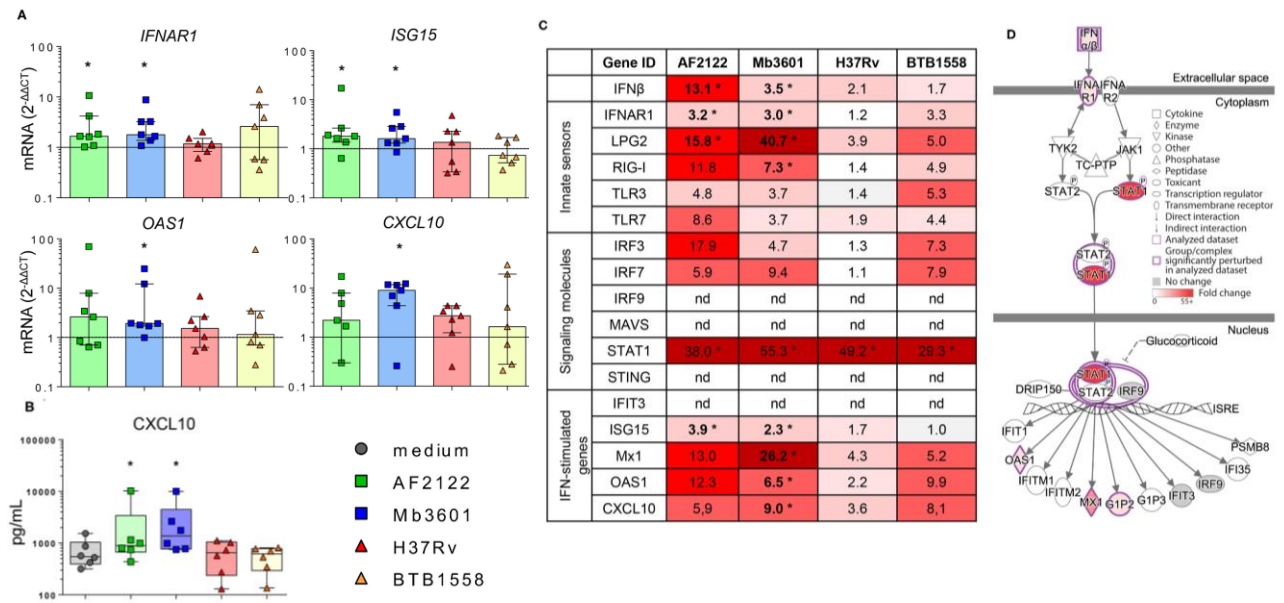


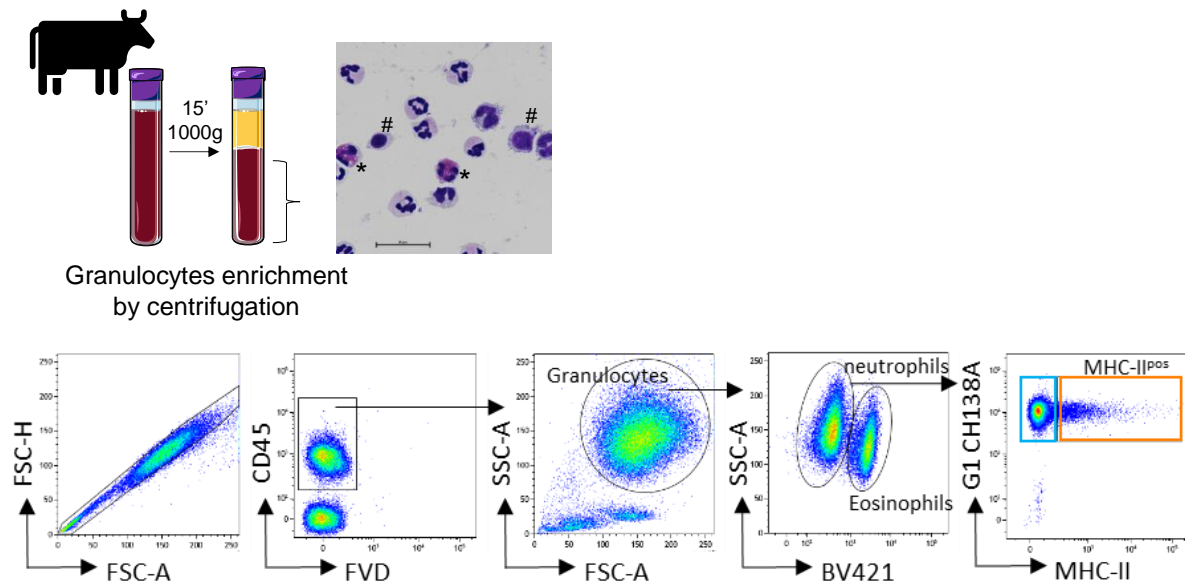
Figure 5 from the article Remot et al., 2021: Mb but not Mtb infection in the lung tissue from Blonde d'Aquitaine cows induces the type I interferon pathway. PCLS were infected as described in Figure 1 of t. (A) IFNAR, ISG15, CXCL10 and OAS1 gene expression at 2 dpi. Individual data and the median and interquartile range in each group are presented (n=7) (B) CXCL10 protein level was measured in PCLS supernatant at 2 dpi. Individual data and the median and interquartile range in each group are presented (n=6). (C) The table represents the mean of fold change (2-ddCT) for each group (n=7) of major genes involved in type I interferon pathway. Graduated red box coloring are for higher gene expression and asterisks mark significant differences compared to uninfected PCLS. nd=not detected. (D) Ingenuity Pathway Analysis drawing of the Type I interferon pathway under IFNAR in the Mb3601 group. Graduated red box coloring are for higher gene expression. (A, B and C) \* p<0.05 (Wilcoxon non-parametric test).

The PCLS model in cattle is a valuable tool to deepen our understanding of early interactions between lung host cells and mycobacteria. It revealed striking differences between cattle breeds and mycobacterial strains. This model could help deciphering biomarkers of resistance *versus* susceptibility to bTB in cattle as such information is still critically needed for bovine genetic selection programs and would greatly help the global effort to eradicate bTB.

#### iv. First characterization of bovine neutrophil subsets

**Neutrophils** are key immune cells. Long considered as “suicide bombers” these phagocytes, that are instrumental during innate immunity, were upgraded as global players of the immune response along with the discovery of new functions. In addition to their unique antimicrobial properties, they fully participate in shaping the adaptive immunity by cross-talk with other cells, and suppressing T-cell proliferation and activity (Müller et al., 2009). They can also migrate to draining lymph nodes, like DCs or inflammatory monocytes, and can shuttle live microorganisms (Abadie et al., 2005). This broad range of phenotypes and functions was recently linked to discrete populations of neutrophils characterized by unbiased single-cell analyses (Lyadova, 2017). These new roles are now documented for humans and mouse in

inflammatory environment (Chatfield et al., 2018). Learning from the results in the mouse model obtained in the laboratory by Dr. Doz-Deblauwe, we recently conducted a thorough characterization of neutrophils circulating in cattle blood under normal conditions. This work was initiated with the BoNeuro project (2018-2019), funded by the Institut Carnot France Future Elevage, which I coordinated.



Two populations of neutrophils can be distinguished on the basis of specific surface makers, including MHC-II (Figure adapted from Rambault et al 2021a et Rambault et al., submitted). Cells were prepared from cattle blood and neutrophils were sorted with a MoFlo AstriosEQ apparatus after labelling with anti-CD45, eFluor fixable viability dye 780 (FDV), anti-G1 (clone CH138A) and anti-MHC-II (clone CAT82A). Eosinophils were excluded based on their autofluorescence in the channel BV421. A representative plot shows the major proportion of G1+ MHC-II<sup>neg</sup> (blue rectangle) and the minor proportion of MHC-II<sup>pos</sup> neutrophils (orange rectangle).

By mimicking -to the closest achievable- the flow cytometry panel and gating strategy from the mouse (Dr. Doz-Deblauwe), we identified a new MHCII expressing subset of regulatory neutrophils that circulate in cattle blood under steady state conditions (Rambault et al., 2021a). These neutrophils are morphologically indistinguishable from classical MHC-II<sup>neg</sup> neutrophils. However, MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils display distinct transcriptomic profiles as illustrated below.

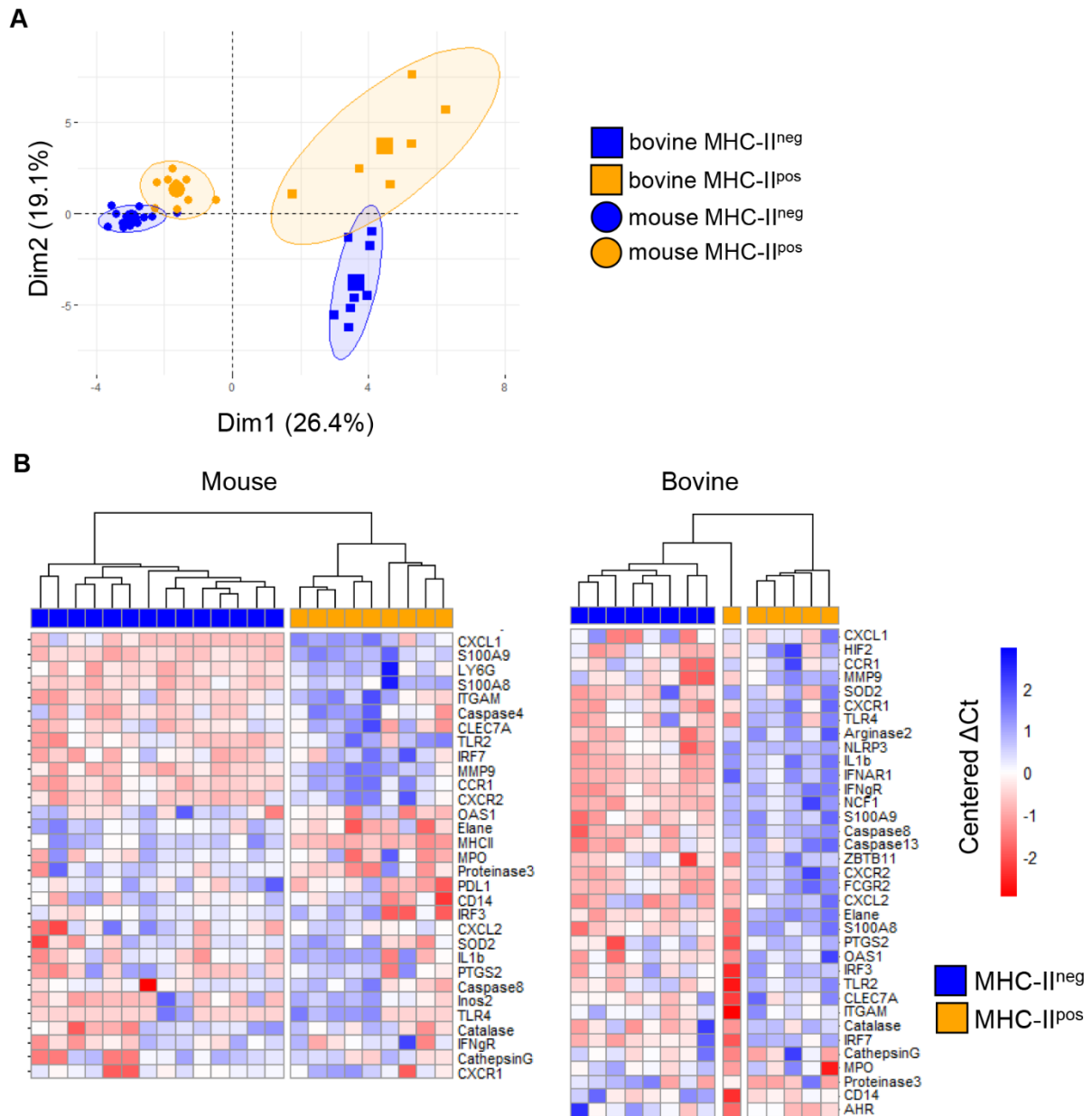
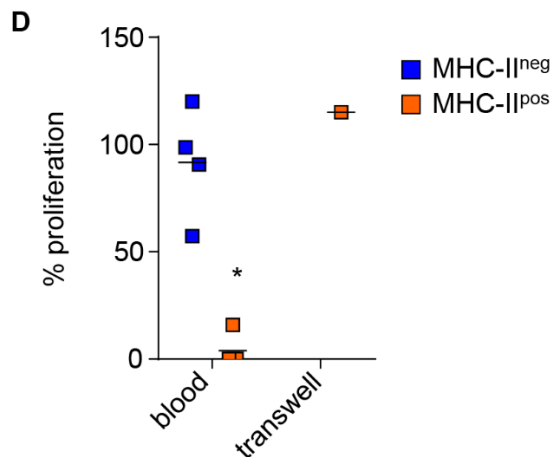
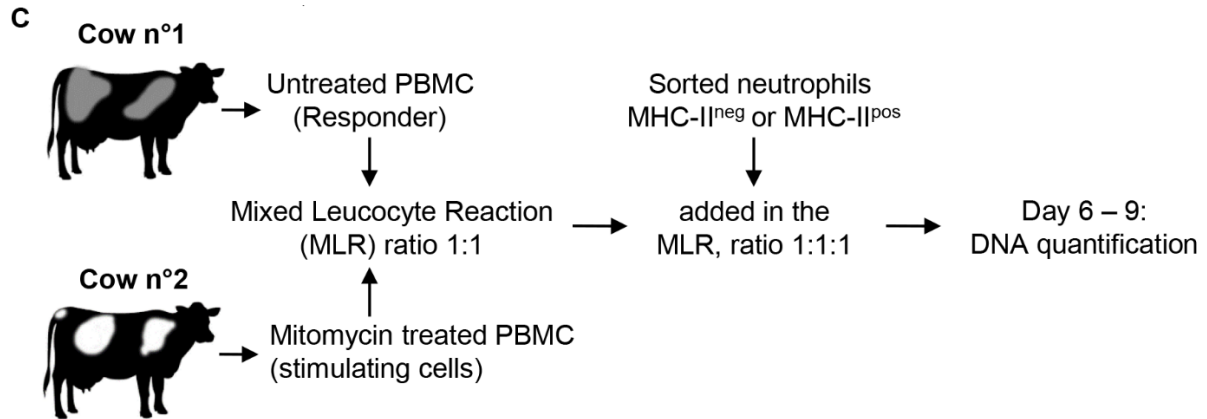


Figure 4 from Rambault et al., 2021a. MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils display distinct transcriptomic signatures. Gene expression of the two purified neutrophil subsets (more than 99% purity) from the two species was assessed by quantitative Real Time PCR using sets of primer pairs designed to cover a large range of neutrophils functions. mRNA expression was normalized to the expression of three housekeeping genes for both species to calculate the  $\Delta C_t$  values (A). Principal Component Analysis (PCA) was performed on  $\Delta C_t$  values and the two first dimensions of the PCA plot are depicted. Inclusion in groups of sets of samples are delineated in the colored areas on the graph with a confidence level of 90% (B) Hierarchical clustering of gene expression was performed on median centered  $\Delta C_t$  values for mouse and bovine data sets, using the ward.D2 method. For each gene,  $\Delta C_t$  values were centered to the median  $\Delta C_t$  value. Higher or lower  $\Delta C_t$  expression compared to the median value were represented respectively in deep to light red or blue. Expression of a selected set of genes indicated on the figure was clearly distinct in MHC-II<sup>pos</sup> neutrophils or MHC-II<sup>neg</sup> neutrophils. Data represent individual samples (Mouse: n= 14 for MHC-II<sup>neg</sup>, n=9 for MHC-II<sup>pos</sup> Bovine: n=8 for MHC-II<sup>neg</sup>, n=6 for bovine MHC-II<sup>pos</sup>).



While sorted MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils displayed similar bacterial phagocytosis or killing activity, MHC-II<sup>pos</sup> produced higher level of ROS and were able to suppress T cell proliferation under contact-dependent mechanisms. Those regulatory neutrophils were highly enriched in lymphoid organs as compared to their MHC-II<sup>neg</sup> counterparts (and in the mouse they express PDL-1, an immune checkpoint involved in T-cell blockade).



Panel C and D of the 6<sup>th</sup> Figure from Rambault et al., 2021a. MHC-II<sup>pos</sup> but not MHC-II<sup>neg</sup> neutrophils exert suppression of T cells at steady state. **(C)**. For the bovine, a Mixed Leucocyte Reaction (MLR) was set up to assess polyclonal T cell proliferation. PBMCs from the responder animal were isolated and left untreated, while PBMCs from the stimulating animal were incubated with mitomycin C. PBMCs from the two cows were incubated at ratio of 1:1. To assess the impact of neutrophils on cattle T cell proliferation, 10<sup>5</sup> of sorted MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils from the responder animal were added to the reaction at day 4. DNA was quantified at day 6 and 9. **(D)** The proliferation was calculated by subtracting DNA values at day 9 from day 6 values. Proliferation measured in the MLR without neutrophils was defined as the reference 100% proliferation. The proliferation observed in the presence of sorted MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils was calculated according to the DNA content (Day 9 – Day 6) and expressed as percentage of the reference value. In one experiment, MHC-II<sup>pos</sup> neutrophils were separated from proliferating cells by a Transwell device. Individual data and the median in each group are represented (3 independent experiments, n=4 cows, n=1 cow for transwell experiment; each represented value is the mean of technical triplicates). \*, P<0.05 Mann Whitney non parametric test.

To sum up, we characterized **two neutrophil subsets and discovered - for the first time in cattle - a new population of regulatory neutrophils that expresses MHCII and displays suppressive activity on T-cells** (Rambault et al., 2021a). Our results emphasize neutrophils as true partners of the adaptive immune response, including in domestic species. They open the way to the discovery of new biomarkers and therapeutic interventions to better control cattle diseases.

We believe that the diversity of phenotypes and functions of neutrophils needs to be investigated in more detail and we advocate that the classical description of neutrophils as a homogeneous cell type in cattle must be revisited. The function of MHC-IIpos neutrophils during the course of infections remains unknown and this topic is my current focus and perspective for the coming years. The interactions between neutrophils and Mb are discussed in the perspectives' section, since this project has just been launched (ANR JCJC Neutro\_bTB 2022-2025).

#### v. Mastitis: context and state of the art

Dairy production holds a special place in French agriculture and agri-food industry. Its major economic weight of 30 billion euros makes it the second agri-food industry after the meat sector and generates a positive trade balance of 3.2 billion euros<sup>1</sup>. **Mastitis** are infection of the mammary gland mainly caused by bacteria. Clinical mastitis are diagnosed with local signs of inflammation (redness, painful and warm udder) and visible lumps in milk. Subclinical infections are defined as inflammation where milk somatic cell count (SCC) exceed 300,000 cells/mL, without any visible clinical signs. Due to its high prevalence (20% of cows will develop a clinical mastitis during their lactation, and a third of cows' udder quarters have a subclinical mastitis), mastitis affect the health and welfare of animals and leads to economic losses due to lower milk production and quality, antibiotic treatments and premature drying off and culling (Gonçalves et al., 2017; Halasa et al., 2007).

The increase of the SCC during mastitis is mainly due to **the rapid influx of neutrophils** from blood to the mammary gland (Koess and Hamann, 2008; Rainard and Riollet, 2003; Schwarz et al., 2011). Neutrophils become the major cell type in inflammatory milk, before macrophages and lymphocytes (Rambault et al., 2021b). Nowadays, **differential leukocytes count** is investigated as a **new tool for mastitis diagnosis** (Damm et al., 2017; Gonçalves et al., 2017; Schwarz et al., 2011). The hypothesis is that the proportion of leukocytes populations (neutrophil/macrophage ratio) provides more precise information about the inflammatory status of the mammary gland, but the description of cell types is limited to total neutrophils, macrophages and lymphocytes with no information about subsets (Godden et al., 2017; Kirkeby et al., 2020; Pilla et al., 2012). A better understanding of the involvement of neutrophils in mastitis would improve differential counts and pave the way for the identification of potential biomarkers of mastitis.

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<sup>1</sup> CNIEL, *L'Economie Laitière en Chiffres*. 2018

- vi. Deciphering the role of neutrophils subsets in the context of clinical and subclinical mastitis (Masticells project and Marion Rambault’s PhD project, 2019-2023)

Mastitis is still one of the most important issue for dairy farming; thus, investigating **how MHC-II<sup>POS</sup> neutrophils behave during mastitis** would bring new views on the physiopathology of this costly disease. Capitalizing from the BoNeutro results, our team obtained a grant from APIS-GENE for the Masticells project (coordinated by Dr. P. Germon, I am the leader of the neutrophil WP). We also secured a CIFRE PhD fellowship for Marion Rambault (who did her Master2 internship on the BoNeutro project under my supervision). For her PhD project, Marion Rambault succeeded to set up protocols to work with **inflammatory milk** and optimized multiple flow cytometry panels. She analyzed **neutrophil recruitment in dairy cow during clinical and subclinical mastitis** (Rambault et al., 2022). Her results highlights are:

- MHC-IIpos neutrophils similarly circulate in blood of healthy Holstein and Normande cows
- Unsorted MHC-IIpos neutrophils display higher phagocytic capacity and ROS production than MHC-IIneg neutrophils
- MHC-IIpos neutrophils with high microbicidal activities are recruited to the mammary gland
- Ratios of MHC-IIpos and MHC-IIneg neutrophils circulating in blood and recruited to milk do not vary during the course of a clinical mastitis episode (d0, d4 and d21 post diagnostic)
- MHC-IIpos regulatory neutrophils are present in milk during subclinical mastitis and positively correlate with T cells (figures below)

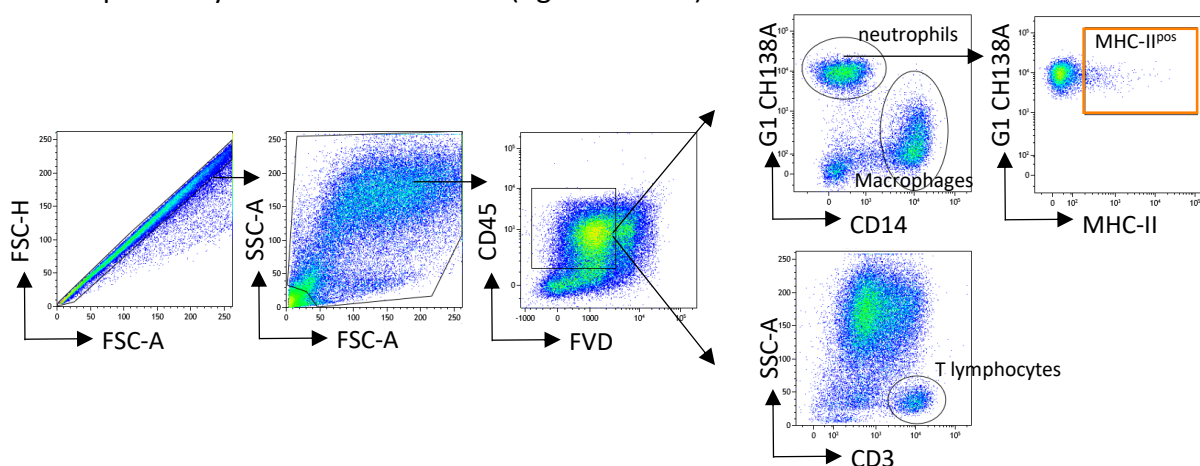


Figure adapted from Rambault et al., 2022 submitted: Flow cytometry gating strategy used to identify different cells populations in milk. Milk samples were diluted in Alsever buffer and centrifuged to remove the cream. Cells were washed several times in PBS 2% fetal calf serum and EDTA before the labelling with primary antibodies and then appropriated conjugated secondary antibodies. Milk neutrophils, macrophages, and T lymphocytes are defined as CD45+ G1+ CD14-, CD45+ G1- CD14+ and CD45+ CD3+ respectively. Milk MHC-IIpos neutrophils are defined as MHC-II+ in the “neutrophils” G1+ gate.

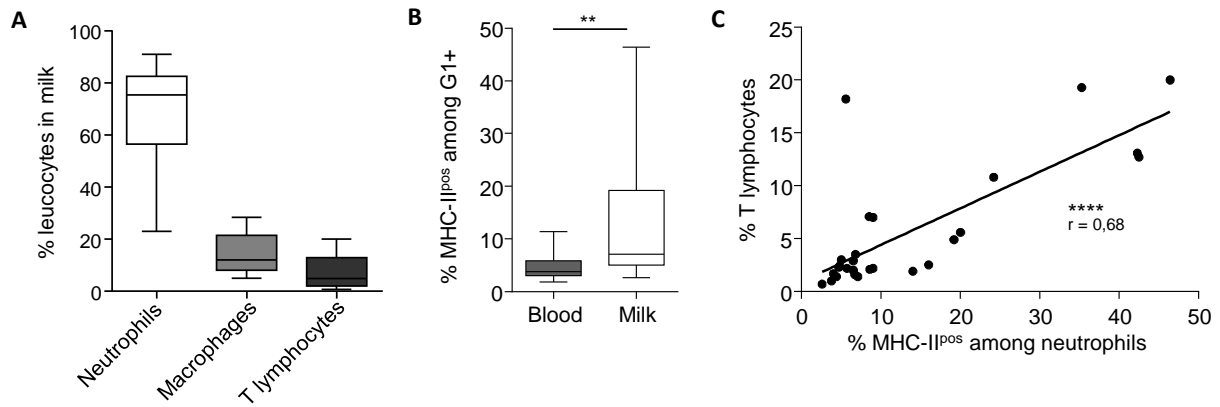


Figure from Rambault et al., submitted: MHC-II<sup>pos</sup> neutrophils accumulate in milk during subclinical mastitis when T cells are also present. (A) Percentage of neutrophils, macrophages and T lymphocytes in milk during subclinical mastitis. (B) Percentage of MHC-II<sup>pos</sup> neutrophils in blood and in milk during subclinical mastitis. (A, B) Data are boxes and whiskers min to max. The gating strategy is described in the previous figure. (C) Correlation between the percentage of T lymphocytes and MHC-II<sup>pos</sup> PMN in milk during subclinical mastitis. Data represent n = 2 independant experiements with n = 27 quarters with SSC > 150000 cells/mL from 17 cows. \*\*P<0.01; \*\*\*\*P<0.0001 (Wilcoxon signed-rank test) (B), Spearman correlation test with 95% confidential interval (C)).

This work is available in BioRxiv (Rambault et al., 2022) and submitted to Journal of Dairy Science (Marion is first and I am last author).

Our study suggests that **new tools such as differential counting used for mastitis diagnosis can be improved** with a more accurate characterization of neutrophil subsets. This could also open the way to the discovery of **new biomarkers of chronic inflammation**.

#### D. Grants

The table here after recapitulates **my role in all the projects funded** since I obtained my INRAE permanent position. **Blue and yellow boxes stand for past and ongoing projects respectively.**

I also participated to the conception and writing of **2 research projects submitted**:

- Fer-TB-Vax project, submitted to the Carnot France Future Elevage, which aim to use the ferret as a model for future vaccination trials against animal tuberculosis in wild life (INRAE/ANSES collaboration).
- TB-Adapt project, submitted to the European ICRAD call, which ambitions to define the molecular determinants of Mycobacterial adaptation and host/pathogen Interaction to inform bTB control (collaboration between: The UK Royal Vet College; Birkbeck University of London; UCD; INRAE and Argentina national institute of Agricultural technology)

Below is the list of **rejected project** for which I contributed significantly to the conception/writing/obtention of preliminary data:

- H2020 ERA-NET, ICRAD, AAP 2020 “TB-GAP: Defining granulomas to advance interventions in cattle and wildlife tuberculosis”. I participated to meetings to elaborate the project and wrote tasks for one WP. Coordinator: Sandrine Lesellier (ANSES)
- H2020 Innovative Action, SFS-11 topic Antimicrobials and animal production, AAP 2019 “ATOMIC: Combating antibiotic resistance by validating novel Alternative compounds TO antiMICrobials used to prevent diseases. Improve animal efficiency and welfare and increase environmental sustainability of livestock farming”. I participated to meetings to elaborate 1 WP inside the project. Coordinator: Paolo Ajmone Marsan (Università Cattolica Del Sacro Cuore, Italy)
- ANR AAP 2016 “bTB Spread: Molecular traits and Host response linked to bovine tuberculosis multispecies transmission”. I participated to meetings to elaborate the project and reviewed it. Coordinator: Marie-laura Boschioli (ANSES)
- ANR AAP 2016 “High macrophage TGF- $\beta$ 2 levels underpin disease susceptibility of Holstein-Friesian cattle to diverse intra-cellular pathogens” I obtained preliminary data, I wrote one section of the project and made the figures. Coordinator: Gordon Langsley (INSERM)
- Institut Carnot France Future Elevage AAP 2016. Projet “BASILICA: Biomarkers of Infectious Lung diseases in Cattle”. I obtained preliminary data and wrote the project. I was the coordinator for this proposal.

Person. month	Call, funding agency	Acronym & Project's title	Name of the scientific coordinator	Start - End	My role
6	Young researcher 2015, INRAE, Animal Health division.	BIIMbo: Bovine Innate Immunity against <i>Mycobacterium bovis</i>	Aude Remot	2016 - 2017	<b>Coordinator</b>
2	EGER 3.0 2017-2021 APIS-GENE	HealthyCalf: Calf health and innate immune biomarkers of susceptibility to infections	Fabrice Laurent (INRAE)	2017-2021	WP4 leader
6	Young researcher 2017, INRAE, Microbiology division.	LIMBO: Lung innate immunity against <i>Mycobacterium Bovis</i>	Aude Remot	2017 - 2018	<b>Coordinator</b>
6	Institut Carnot	BoNeuro: Bovine Neutrophil, a biomarker of robustness against mastitis	Aude Remot	2018-2019	<b>Coordinator</b>
3	Hubert Curien Program (PHC Ulysses)	ONE-TB: the study of bovine tuberculosis through a One Health approach	Aude Remot & Stephen Gordon (UCD)	2018	<b>Co-Coordinator</b>
3	ANR AAP2017 Défi 5, Axe 2 - PRC	EpiLungCell: Establishment of <u>Epithelial Lung cell</u> lines for pathogens investigation	Bertrand Pain (INSERM)	2018 - 2021	In charge of 2 tasks
0.5	INRAE Animal Health division	MetaBov: Immunometabolic signatures of <u>bovine</u> innate cells	Delphine Descamps (INRAE)	2020 - 2022	In charge of all neutrophil related work
0.5	FEDER, APR-IA 2019 Région Centre Val de Loire	ANIMALT: Reducing the number of animals in research, development of alternative <i>ex-vivo</i> models.	Sonia LACROIX-LAMANDE (INRAE)	2019 - 2022	In charge of bovine lung experiments
1.5 (less after 2021)	FEDER, Program Centre-Val de Loire 2014-2020	IMAG'ISP: Imaging in infectiology	Nathalie Winter (INRAE)	2019 - 2022	Bovine lung/cell imaging
2	Hubert Curien Program (PHC Ulysses)	BoNeuViD: Immunomodulation of bovine neutrophils by Vitamin D for control of <i>Mycobacterium bovis</i> infection	Aude Remot & Kieran Meade (UCD)	2021 - 2022	<b>Co-Coordinator</b>
2	EGER 4.0 2019-2022 APIS-GENE	MASTICELL: Phenotypic and functional characterization of milk somatic cells involved in mastitis responses	Pierre GERMON (INRAE)	2019 - 2023	WP2 leader, co-supervisor of PhD Student
1 (less after 2021)	EU Horizon 2020. INFRAIA-01-2016-2017 call	VetBioNet: Veterinary Biocontained facility Network for excellence in animal infectious disease research and experimentation	Sascha TRAPP (INRAE)	2017 - 2023	In charge of bovine lung experiments
0.75	PSPC Pays de Loire, BPI France	Néolac : Nouveaux produits pour la rEduction des antibiOtiques, la durabilité de la filière LAitière et sa Compétitivité	Gaëtan Vétea Plichart (Biodevas Laboratory)	2020 - 2023	In charge of all neutrophil related work
1	Fédération de recherche en Infectiologie (FéRI)	MyCOVID: Comparison of innate lung responses after <i>ex vivo</i> infection by mycobacteria from the <i>Mycobacterium tuberculosis</i> complex and the SARS-CoV-2	Aude Remot & Sébastien Eymieux (INSERM, CHU)	2022 - 2023	<b>Co-Coordinator</b>
6 (more after 2023)	ANR AAP2021, CE20, JCJC	Neuro-bTB: Deciphering the role of bovine neutrophil subsets in bovine tuberculosis	Aude Remot	2022 - 2025	<b>Coordinator</b>

## 5. Mentoring

Inspiring future generation and creating a vibrant research climate is very important to me. Mentoring students and young collaborators is a mission which I hold dear in my heart. I train students each year and try to keep track of each person whom I mentored. I gladly help them to pursue their carrier with advice and recommendations.

For the first time, I am co-supervisor of a PhD Student, Marion Rambault, with the help and supervision of Nathalie Winter. I guide Marion through her PhD, on a scientific point of view, but I also help her to gain skills, build a network and a career in research.

The table below presents the students I mentor(ed), and how they carried on after their internship.

Level of study	Name	Period	Project	Current position
<b>PhD Student</b> (CIFRE contract)	Marion Rambault	Nov 2019- Feb 2023	Role of neutrophils in mastitis in dairy cows	PhD student (viva 15th December 2022). Marion wants to pursue a research career and is looking for a post-doctoral position.
<b>Master 2 student</b> Tours university		Jan – Jun 2019	Phenotypic and functional characterization or bovine neutrophils	
<b>Master 2 student</b> Tours university	Théodore Vinais	Jan – Jun 2018	Interactions between bovine neutrophils and <i>Mycobacterium bovis</i>	Theodore is now project manager at the Esquirol hospital in Limoges
<b>Master 2, Paris V, VII, XI and XII</b>	Azadeh Djavid	Jan-Jun 2010	PAR and TLR expressions in lambs (I co-supervised the practical work only)	Medical Doctoral, Azadeh is now pediatrician in Paris
<b>Master 1 student</b> Tours university	Marion Horta	Apr-May 2021	Infection of human PCLS with Mb and Mtb	Marion is now in Master 2, Tours university
<b>Master 1 student</b> Tours university	Alexia Tessier	Jun-Jul 2021	Role of neutrophils in mastitis in dairy cow (Marion Rambault co- supervised Alexia's internship)	Obtained her Master2 in Jun2022, is looking for a job in R&D
<b>Master 1 student</b> Tours university	Leslie Hertereau	Apr-May 2021	Transcriptomic signatures of calf alveolar macrophages infected with BCG	Obtained her Master2 in Jun2022, and obtained a 2 years research engineer position in a public laboratory



<b>Master 1 student</b> Tours university	Florian Tomal	Apr-May 2017	Interactions between bovine alveolar macrophages and <i>Mycobacterium bovis</i>	Currently pursuing his PhD in the ISP unit (team AIM, CIFRE Contract)
<b>Master 1 student</b> Paris-Saclay university	Amélie Riou	Apr-May 2015	Isolation and screening of bacteria from broncho-alveolar lavages from allergic children	Obtained her Master in 2016 – now senior consultant (Strategic and industrial transformation)
<b>Professional Bachelor student</b> (3rd Year), Tours university	Anthony Coupé	Feb-May 2021	Interactions between lung epithelial cells and <i>Mycobacterium bovis</i> .	Obtained a technician position at Tours hospital just after his internship
<b>Bachelor Student</b> (2 <sup>nd</sup> Year) Orsay University	Alix Penel	Apr-May 2014	Isolation and screening of bacteria from SPF neonatal mice lungs	Alix did a Master, worked few years in Paris Hospitals as a research assistant and is now working for the Foundation Adolphe de Rothschild (managing clinical research project)
<b>DUT student</b> (University Diploma of Technology) Orsay University	Nicolas Garcelon	Apr-May 2015	Isolation and screening of bacteria from SPF neonatal mice lungs	Nicolas did a Business School after his Science Bachelor and is now consultant in management at Transformation Factory
<b>Research assistant</b> (temporary worker)	Elliot Mathieu	Sep-Nov 2015	Crosstalk between lung microbiota and lung cells in mice	Elliot did a PhD in the team (CIFRE contract) and is now Production manager in functional metagenomics at MetaGenoPolis
<b>Veterinary student</b> (3 <sup>rd</sup> year) University college Dublin	Roxane Pépin-Donat	Jun 2019	Analysis of lung explants responses to various TLR ligands	DMV internship, Touraine Intensive care unit

I also gladly welcome students for short visits. For instance; Marion Bouchier and Elisa Contat (VetAgro SUP, ENVL) visited one week in July 2019, they defended their veterinary thesis in December the same year. In the frame of the PHC Ulysses projects (INRAE/UCD), I welcomed Bojan Stokjovic in 2018 for one week during his post-doctorate (Bojan is now doing another post-doctorate in Australia). In 2021, I welcomed Susana Flores-Villalva for 7 weeks, we performed neutrophil experiments together and I am associated to 2 publications submitted to the journal Veterinary Immunology and Immunopathology (role of vitamin D for the control of mycobacteria). Susana will defend her PhD this autumn. In 2022, I welcomed Angelique



Corset for 3 weeks (ELISA and RT-qPCR Fluidigm), Angelique is doing her PhD in the frame of the Neolac project.

My ambition is to stimulate a research interest in the new generation of scientists, and especially encourage women. The Agreenskills EU mobility during my post-doctorate was very instructive and helpful for my carrier, I would like to encourage young French scientists to consider international fellowships, and I hope that I will have the opportunity to welcome foreign students in the future. This year, I am capitalizing on my ANR Neuro-bTB project and preliminary results to propose a PhD position for 2023.

## 6. Teaching and evaluation activities

Although my ambition was always to become a researcher, I like to teach immunology to students from different grades. I am committed to take time to teach according to the coming opportunities.

I did 2 lectures during my PhD (for students in 3<sup>rd</sup> year in AgroParisTech) and during my post-doctorate I was co-responsible of the molecular immunology teaching unit (together with Dr. D. Decamps). I taught 26h in total to Master1 students at Cergy Pontoise university. I also mentored 3<sup>rd</sup> year med students across bibliography and project's conception and presentation (CHU Saint Antoine, Paris).

In 2015 I obtained the French "qualifications"<sup>2</sup> in the section n°65 (cellular biology) and n°66 (physiology).

Since my recruitment, I teach students at the University of Tours. I am doing one lecture per year on mycobacterial immunity (1h30) for Master2 students in Infectiology at Tours university (in English, lecture for both the national Master I2VB and the international IDOH Erasmus Master students). Last year I also gave a practical course on bovine lung explants to IDOH students (1/2 day, to be repeated each year).

In June 2022, I introduced Science and Immunology to children between 3 to 12 years old, in the kindergarten and elementary school of Semblancay, near Tours. This intervention was part of an event organized by the French Society of Immunology, to promote science curiosity for the youngest.

In addition to occasional teaching, I also provide my expertise as jury members every year for Master 1 and Master 2 students' thesis review and defenses.

I am also solicited for PhD committees (Comité de Suivi Individuel), so far, I followed:

- Damien Garrido, who worked on chicken immunity to influenza viruses and avian *E. coli*. He defended his PhD at the University of Tours in 2018.
- Rea Bingula, who worked on the gut-lung axis in the context of lung cancer and

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<sup>2</sup> Mandatory to obtain a professor position in a University in France

stimulation with prebiotic. She obtained her PhD at the university of Clermont Auvergne in 2019.

- Maud Rouault, who is working at ONIRIS (Nantes veterinary school) on the identification of calves' immunological biomarkers of sensitivity and severity to bronchopneumonia. She started her PhD in December 2021, and we met for her 1<sup>st</sup> committee in June.
- Angélique Corset, who is working in Rennes (INRAE St Gilles & Biodevas company) on nutritional strategies to modulate the anti-oxidant and immune status of dairy cows. She started her PhD in August 2021 and the first PhD committee was in Nouzilly in June 2022 when Angélique came 3 weeks to perform experiments in our team.

I was member of PhD's jury two times, I was examiner for the Science PhD of Rea Ringula in 2019 (Influence lung cancer via prebiotical stimulation of the gut microbiota), and reviewer for the Pharmacy thesis of Myriam Slimi in 2017 (Properties and characteristics of the microbiota, the causes of dysbiosis and the impact on cardiovascular risk factors such as obesity and hypertension).

## 7. Perspectives

For the coming years, my main focus will be the analysis of the role of bovine neutrophil subsets. I secured an ANR JCJC contract on this topic, and I just submitted a PhD application to obtain a fellowship for 2023 (INRAE/Region Centre or national grant MRT).

### Deciphering the role of neutrophil subsets in the pathogenesis of bTB (ANRJCJC Neuro\_bTB 2022-2025)

**Early interactions** between cattle neutrophil and Mb, as well as neutrophil interactions with other cell types are key for bacilli elimination before the onset of the adaptive response, as observed in humans (Martineau et al., 2007) and could also explain why in herds some heavily exposed animals do not develop positive skin test (Cassidy and Martineau, 2014). At **later phases** of infection i.e. when lesions (granulomas) are already established at onset of adaptive immunity, animals generally become TST positive and reactors must be eliminated by law in Europe. However, depending on bacilli constraint by the granuloma, animals may have shed bacilli in the field before withdrawal. Neutrophils could also play important role in the development of granuloma as shown in human TB. My colleagues and I were invited to publish a review in *Frontiers in Immunology*, and discussed the key role of neutrophils and changes in the hypoxic environment in the context of TB (Remot et al., 2019). Neutrophils can also impact bacilli shedding in the environment during bTB (Cassidy, 2006; Palmer et al., 2022).

I hypothesize that the two MHCII<sup>pos</sup> and MHCII<sup>neg</sup> neutrophil subsets that we identified play different roles during Mb infection in cattle. The Neutro-bTB ANR JCJC project to decipher the role of bovine neutrophil subsets in bTB has just started in May 2022.

The project is built in two work packages.

First, I will investigate the **early interactions** between cattle neutrophil subsets and Mb, and tackle neutrophil interactions with other cell types in the lung environment *ex vivo* using the original PCLS model (Remot et al., 2021). We hypothesize that MHCII<sup>+</sup> and MHCII<sup>-</sup> neutrophils will behave differently in response to Mb infection and / or to other cells from their close environment in the lung (Doz et al., 2013; Lombard et al., 2016).

WP1 will address the two key questions:

- How do bovine neutrophils respond to and kill Mb? Are the two subsets different?
- How does the cattle lung environment activate neutrophils? Do the two subsets respond differently?

Second, I will decipher the roles that neutrophils play **at later phases** of infection when CD4<sup>+</sup> T cells and  $\gamma\delta$  T cells producing IFN $\gamma$  are recruited to the site of infection. Our hypothesis is that MHCII<sup>pos</sup> neutrophils alter Mb containment whereas MHCII<sup>neg</sup> reduce Mb load and favor granuloma integrity.

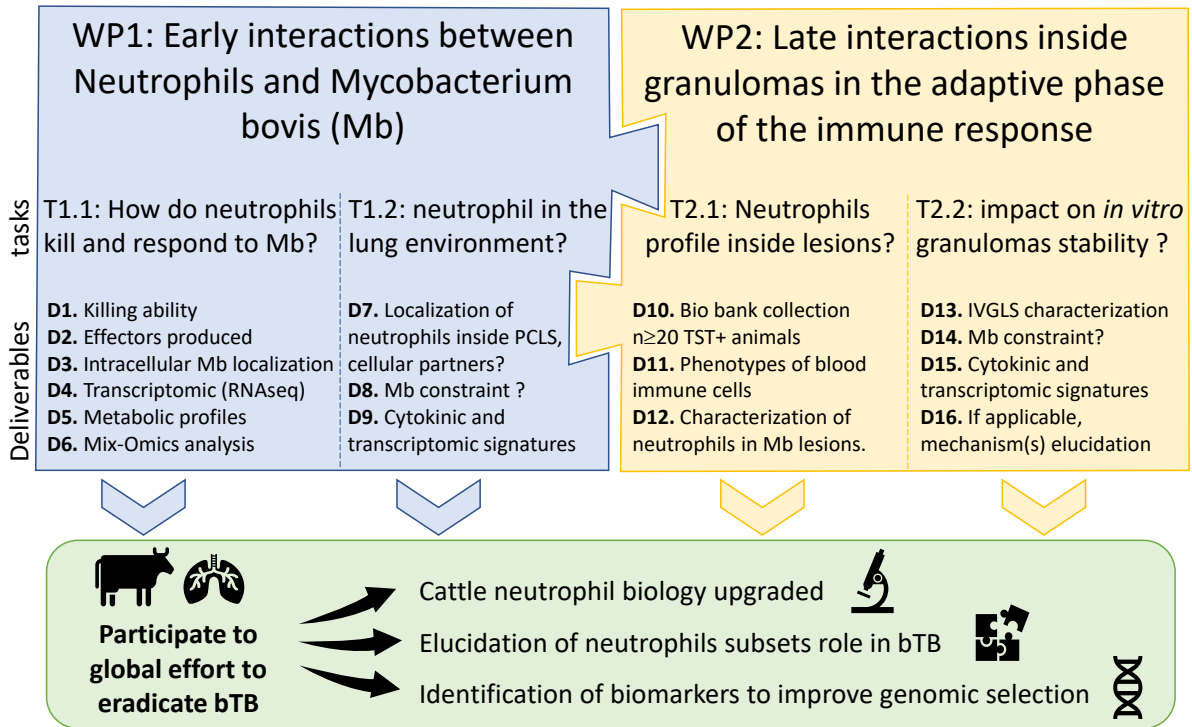
WP2 will address the following key questions:

- What is the profile of neutrophils inside bTB lesions in cattle?

- Do MHCII<sup>POS</sup> neutrophils alter granuloma stability?

For this WP I have established the necessary collaborations with Pr. V. Blanquet (Univ Limoges) to get access to field samples from Mb contaminated animals and with Pr. A. Dorhoi (Friederichs Loeffler Institute, Germany) who established the *in vitro* granuloma model.

### Neutro-bTB: Deciphering the role of bovine neutrophil subsets in bovine tuberculosis



Workflow of Neutro-bTB project. The deliverables are summarized under each task.

bTB eradication is an unmet priority. The poor understanding of bTB pathophysiology in cattle and the lack of correlates of protection are substantial knowledge gaps that must be resolved so as to better tackle the disease. Neutro-bTB project will decipher the role of newly discovered neutrophils subsets in the pathogenesis of bTB in cattle. Genomic selection has been very successful in cattle and is a promising way to improve cattle Health in the context of infectious diseases (for instance the genetic variability of mastitis resistance is well established in dairy cattle) (Boichard et al., 2016; Rupp and Boichard, 2003). We believe that deciphering the role of neutrophil subsets in bTB could lead to the discovery of new biomarkers that could improve cattle genomic selection program. In humans, genetic control of TB susceptibility or resistance is multifactorial. Although cells involved in clinical outcome of TB infection remain incompletely elucidated, neutrophils are prominent candidates (Kroon et al., 2018). Therefore, neutro-bTB results could help deciphering biomarkers of resistance versus susceptibility to bTB in cattle. As such information is still critically needed for bovine genetic selection programs, the Neutro-bTB project could greatly help the global effort to eradicate bTB.

TST that is the basis of bTB control in cattle is the signature of productive infection and becomes positive on the onset of adaptive immunity when primed T-cells produce IFN $\gamma$  in

response to mycobacterial antigens. If cattle were to be selected for improved innate resistance to Mb infection – possibly through more effective neutrophils - cattle encountering Mb would eliminate bacilli and remain negative for TST. This would result in less culling of animals and less loss for the breeders which would greatly benefit breeders and animal health as well as the economy. Improving resistance to bTB could also result in less Mb shedding in livestock.

Neutrophils are key immune cells for early elimination of a large range of invading pathogens in cattle. Our results will also be a great advance for veterinary immunology as they will not only provide a better understanding of bTB pathogenesis but also contribute to a better understanding of cattle neutrophil biology in general.

### Neutrophils in other pathologies / mastitis

Better comprehension of how the immune response is locally orchestrated in response to respiratory infection will also benefit to cattle health. **Bovine respiratory disease complex** is an economically important disease of cattle as animals are sensitive to a variety of respiratory viruses and bacteria especially during transport (Earley et al., 2017). Neutrophils are highly involved in the pathogenesis of this complex disease (Murray et al., 2016). The new tools developed the Neutro\_bTB project will bring important advances to the comprehension and future treatment of bovine respiratory diseases (and possibly lead to new collaborations).

Our results will also apply to **mastitis** management. It is well established - especially by the IBIR team - that early and well controlled influx of neutrophils to the mammary gland upon invasion by a pathogen is the prevalent mechanism for protection (Rainard and Riollet, 2003). The Masticells project ends in 2023 but another project might be submitted to APISGENE to continue our investigation, potentially a focus on interactions between immune cells and *Streptococcus Uberis*.

I am currently involved in another research project which tackles the effect of a new nutritional product on cattle Health, via the modulation of immune cells including neutrophils. The **Neolac project** just started in August 2021 and is funded by BPI-France, Région Pays de la Loire et Bretagne. This vast project aims to deliver new nutritional products to reduce the use of antibiotics in dairy cows and improve milk quality. The consortium is composed of:

- Biodevas: The company (small and medium size business) producing plant-derived products which are tested as nutritional additives for dairy cows.
- SODIAAL: the 1<sup>st</sup> dairy cooperative in France, who is piloting field trials in 100 farms, and also control milk quality (and milk derived products) from supplemented animals.
- INRAE: 3 teams from different campus (Rennes, Clermont-Ferrand, Nouzilly) will investigate the mode of action of the products and run two *in vivo* experimental protocols to objectify the effect of the nutritional solutions on the animal Health (following antioxidant status, response to inflammatory challenge etc).

My contribution is to investigate the modulation of immune responses between animal lots, especially neutrophil subsets proportion, production of ROS, and phagocytic capacities.

I found very important to continue to work on both disease (bTB and mastitis), and the role of neutrophils is a very good angle to cultivate transversality in the team.

#### [PCLS: a model which gave me a good visibility and attracts collaborators](#)

During the Covid19 crisis, a research team in Tours hospital reached us to set up **human PCLS experiments**, to investigate interactions between SRASCov2 and lung cells, in a preserved lung environment. We established a workflow, and signed a convention between Tours hospital and INRAE. Our project, entitled Mycovid as a wink to both Mycobacteria (Mb and Mtb) and Covid19, was awarded by 10k€ prize from the Fédération de Recherche en Infectiologie this summer. My collaborators and I have just started to analyze the lung responses to Mb, Mtb and SRAS-Cov2. I lead the mycobacteria part. I aim to compare the results obtained in bovine PCLS infected with Mb vs Mtb; with human PCLS features to address host species jump and host specificities. My collaborators and I ambition to publish our first results next year.

The PCLS model was also a hook to start collaboration with Pr. S. Gordon's team in UCD, who are co-authors in our article (Remot et al., 2021). I presented our results at the international conference on Mb in Galway in June 2022. Another team, working at the university of Aberystwyth, approached me to start working together, and to use our model to screen BCG mutants and elucidate bacterial phenotypes. One of their researchers will come this winter for some experiments, and I should visit them next year to take advantage of their facilities (Seahorse Cell Metabolism Technology). I am looking forward this new collaboration, which increase my network outside France.

## 8. Annexes

This section is an exhaustive list of my publications, patents, posters and oral presentations; and participation to events to promote sciences to the general public.

### A. Publications

For my publications, I grant great respect to my collaborator's contributions. On a daily basis in the laboratory, I indicate in my protocols who participate to each experiment (preparation, realization, analyze etc) to help the decision of authors' positions. All protocols are listed in my lab book, updated regularly, signed by Dr. Nathalie Winter and available for consultation by all my team mates. Students are present in the authors' list if their contribution is significant. We discuss the authors' list and their positions openly during lab meetings, as soon as the first steps of each manuscript's preparation. All authors must read and approve the manuscript before submission.

I am committed to the OpenScience and OpenData policy of INRAE. Top priority is given to open access journals promoting a transparent revision process including the publication of raw data (research integrity and open science policy). In order to favor access to my results and increase scientific exchanges, since 2019, my manuscripts are available as pre-prints in the BioRxiv platform and data are archived in open repositories (HAL INRAE). Raw data are deposited on public data base (Gene Expression Omnibus for instance).

For each article below, I indicate the corresponding period of my career (PhD student / post-doc / INRAE researcher) to better understand my contributions according to the steps of my career.

#### **1 submitted publication (submitted to Journal of Dairy Science, and available in BioRxiv <https://doi.org/10.1101/2022.09.01.506187>)**

1. Neutrophils expressing MHC-II circulate in blood and milk during mastitis and present high microbicidal activity.

Rambault M, Gilbert F.B, Roussel P, Tessier A, David V, Germon P, Winter N, **Remot A.**

Period: INRAE CRCN researcher

Contribution: I obtained grants and conceptualized the research project. I helped Marion Rambault, my PhD students, for some of the experiments presented, and analyzed the data with her. Marion prepared the figures and wrote the manuscript. I reviewed and corrected it with Dr. N. Winter and Dr. P. Germon. My PhD student is co-first author and I am last author.

#### **19 accepted publications (sorted by most recent)**

1. Alternative methods to study lung host-pathogens interactions *in vitro*. (title in French: Méthodes alternatives in vitro pour l'étude des interactions hôte-pathogène du poumon). INRAE Productions animales. *In press*.

**Remot A,** Descamps D, Erny A, Chottin C, Drajac C, Carreras F, Ferret C, Archer F.

Period: INRAE CRCN researcher

Contribution: Based on my lung explant expertise, I was invited to write a review on alternative methods to study host-microbes in the bovine lung. I choose my co-authors, proposed a draft and organized the work between Dr. D. Decamps, Dr. F. Archer and myself; us three wrote this review in French, and prepared the figures. I handled the submission, revision and proof reading (I am corresponding author).

2. A Pulmonary *Lactobacillus murinus* Strain Induces Th17 and ROR $\gamma$ t+ Regulatory T Cells and Reduces Lung Inflammation in Tuberculosis.

J Immunol. 2021 Oct 1;207(7):1857-1870. doi: 10.4049/jimmunol.2001044. Epub 2021 Sep  
Bernard-Raichon L, Colom A, Monard SC, Namouchi A, Cescato M, Garnier H, Leon-Icaza SA, Métais A, Dumas A, Corral D, Ghebrendrias N, Guilloton P, Vérollet C, Hudrisier D, **Remot A**, Langella P, Thomas M, Cougoule C, Neyrolles O, Lugo-Villarino G.

Period: Post-Doctorate / INRAE CRCN researcher

Contribution: I attended meetings at the beginning of Lucie Bernard's PhD and sent some bacterial strains I isolated and characterized during my post-Doctorate, which were important added value to Lucie's immunomodulation strategy. I critically review the manuscript before submission and after revision but did not participate in its preparation.

3. Mycobacterial Infection of Precision-Cut Lung Slices Reveals Type 1 Interferon Pathway Is Locally Induced by *Mycobacterium bovis* but Not *M. tuberculosis* in a Cattle Breed.

Front Vet Sci. 2021 Jul 9;8:696525. doi: 10.3389/fvets.2021.696525.

**Remot A**, Carreras F, Coupé A, Doz-Deblauwe É, Boschioli ML, Browne JA, Marquant Q, Descamps D, Archer F, Aseffa A, Germon P, Gordon SV, Winter N.

Period: INRAE CRCN researcher

Contribution: I obtained grants and conceptualized the research project. I performed all experiments presented, analyzed the data, prepared the figures, and wrote the entire manuscript. I took care of the submissions and revisions (BioRxiv and Frontiers in Veterinary Sciences) and correspondence with all co-authors. I did the final proof reading.

4. Single-Shot Vaccines against Bovine Respiratory Syncytial Virus (BRSV): Comparative Evaluation of Long-Term Protection after Immunization in the Presence of BRSV-Specific Maternal Antibodies.

Vaccines (Basel). 2021 Mar 9;9(3):236. doi: 10.3390/vaccines9030236.

Valarcher JF, Hägglund S, Näslund K, Jouneau L, Malmström E, Boulesteix O, Pinard A, Leguéré D, Deslis A, Gauthier D, Dubuquoy C, Pietralunga V, **Remot A**, Falk A, Shevchenko G, Bergström Lind S, Von Brömssen C, Vargmar K, Zhang B, Kwong PD, Rodriguez MJ, Garcia Duran M, Schwartz-Cornil I, Taylor G, Riffault S.

Period: INRAE CRCN researcher

Contribution: I helped for some experiments performed at the PFIE (INRAE experimental unit dedicated to infectiology in Nouzilly) by Dr. S. Riffault in the frame of the European project SAPHIR. I treated several samples collected post-mortem (lung and broncho-alveolar lavages). I reviewed the manuscript but did not participate in its preparation.

5. Neutrophils Encompass a Regulatory Subset Suppressing T Cells in Apparently Healthy Cattle and Mice.



Front Immunol. 2021 Feb 26;12:625244. doi: 10.3389/fimmu.2021.625244. eCollection 2021. Rambault M, Doz-Deblauwe É, Le Vern Y, Carreras F, Cunha P, Germon P, Rainard P, Winter N, **Remot A**.

Period: INRAE CRCN researcher

Contribution: I obtained grants and conceptualized the research project. I helped my PhD students for most of the bovine experiments presented, analyzed the data with my colleagues. The first-authors prepared the figures, but I wrote the manuscript with Dr. N. Winter. I took care of the submissions and revisions (BioRxiv and Frontiers in Immunology) and correspondence with all co-authors. I did the final proof reading. My PhD student is co-first author and I am last author.

6. The microbiota plays a critical role in the reactivity of lung immune components to innate ligands.

FASEB J. 2021 Apr;35(4):e21348. doi: 10.1096/fj.202002338R.PMID: 33715218

Marquant Q, Laubreton D, Drajac C, Mathieu E, Bouguyon E, Noordine ML, **Remot A**, Riffault S, Thomas M, Descamps D.

Period: Post-Doctorate

Contribution: I was associated to this publication from both Dr. D. Descamps and Dr. M. Thomas for my key role in the initiation and deployment of the research topic during my post-dotorate.

7. Isolation of bovine neutrophils by fluorescence- and magnetic-activated cell sorting.

Methods Mol Biol. 2021;2236:203-217. doi: 10.1007/978-1-0716-1060-2\_16

Rambault M\*, Borkute R\*, Doz-Deblauwe E\*, Le-Vern Y, Winter N, Dorhoi A<sup>£</sup>, **Remot A**<sup>£</sup>. \*co-first authors. <sup>£</sup>co-corresponding authors.

Period: INRAE CRCN researcher

Contribution: I wrote a section of this article (which is published as a book chapter), realized figures n° 1 and 2, and organized the work between the rest of the authors (during the first Covid lock-down). I critically reviewed the manuscript and incorporated my co-authors modifications into the final version before submission. I handled the proof reading. My PhD student is co-first author and I am co-last author.

8. A Single Shot Pre-fusion-Stabilized Bovine RSV F Vaccine is Safe and Effective in Newborn Calves with Maternally Derived Antibodies.

Vaccines, 2020. doi: 10.3390/vaccines8020231.

Riffault S, Hägglund S, Guzman E, Näslund K, Jouneau L, Dubuquoy C, Pietralunga V, Laubreton D, Boulesteix O, Gauthier D, **Remot A**, Boukaridi A, Falk A, Shevchenko G, Lind SB, Vargmar K, Zhang B, Kwong PD, Rodriguez MJ, Duran MG, Schwartz-Cornil I, Eléouët JF, Taylor G, Valarcher JF.

Period: INRAE CRCN researcher

Contribution: I helped for some experiments performed at the PFIE (INRAE experimental unit dedicated to infectiology in Nouzilly) by Dr. S. Riffault in the frame of the European project SAPHIR. I treated several samples collected post-mortem (lung and broncho-alveolar lavages) and helped for a bacterial experiment which was not include in the publication. I reviewed the manuscript but did not participate in its preparation.

9. Neutrophils and Close Relatives in the Hypoxic Environment of the Tuberculous

### Granuloma: New Avenues for Host-Directed Therapies?

Front Immunol. 2019 Mar 12;10:417. doi: 10.3389/fimmu.2019.00417.

**Remot A**, Doz-Deblauwe E, Winter N.

Period: INRAE CRCN researcher

Contribution: Together with my two colleagues, I elaborated the draft of the review after an editorial invitation for a TB-related topic. I wrote two sections (« possible influence of the hypoxic Mtb granuloma on key neutrophil-released mediators » & « neutrophils in TB: many open questions ») and handled the submission, revision and the proof.

### 10. CR3 Engaged by PGL-I Triggers Syk-Calcineurin-NFATc to Rewire the Innate Immune Response in Leprosy.

Front Immunol. 2019 Dec 17;10:2913. doi: 10.3389/fimmu.2019.02913.

Doz-Deblauwe E, Carreras F, Arbues A, **Remot A**, Epardaud M, Malaga W, Mayau V, Prandi J, Astarie-Dequeker C, Guilhot C, Demangel C, Winter N.

Period: INRAE CRCN researcher

Contribution: I helped Dr. E. Doz-Deblauwe for some experiments (*in vitro* infection), participated to meetings (brainstorming and data analyses). I critically reviewed the manuscript but did not take part in its preparation (figures or text).

### 11. Paradigms of the lung microbiota functions in health and more specifically in asthma.

Frontiers in Physiology. 2018. 9:1168. doi: 10.3389/fphys.2018.01168

Mathieu E, Escribano-Vazquez U, Descamps D, Cherbuy C, Langella P, Riffault S, **Remot A**, Thomas M.

Period: post-Doctorate

Contribution: In collaboration with Elliot Mathieu, a young research assistant who I trained before leaving the team, I wrote this review on lung microbiota. I did the draft during my post-doctorate with Dr. M. Thomas but the writing was carried out by Elliot Mathieu after my recruitment in Nouzilly. I interacted with Muriel and Elliot *via* visioconferences, wrote a small part on lung immunity, reviewed the entire manuscript and added some key references. I also helped to define our publication strategy. My contribution (original draft, management of 1st author with M. Thomas, and writing/editing) placed me in before last author position.

### 12. Bacteria isolated from lung modulate asthma susceptibility in mice.

ISME J. 2017 May;11(5):1061-1074. doi: 10.1038/ismej.2016.181.

**Remot A\***, Descamps D, Noordine ML, Boukadiri A, Mathieu E, Robert V, Riffault S, Lambrecht B, Langella P, Hammad H, Thomas M\*. (2017). \*co-corresponding authors.

Period: post-Doctorate.

Contribution: I performed all the experiments presented in the article (both in France and Belgium), prepared all figures and wrote the entire manuscript which correspond to my post-doctorate project. I handled correspondence with all co-authors (I am corresponding author), the submission, revision and the proof. A patent was published before the article (Brevet N° 1650656, PCT/EP2017/051839).

### 13. The microbiota shifts the iron sensing of intestinal cells.

FASEB Journal. 2016. DOI : 10.1096/fj.15-276840

Deschemin, J.-C. \*, Noordine, M.-L. \*, **Remot, A.**, Willemetz, A., Afif, C., Canonne-Hergaux, F., Langella, P., Karim, Z., Vulont, S., Thomas, M., Nicolas, G. (2016). \*co-first authors.

Period: post-Doctorate.

Contribution: This study focused on iron and highlighting new aspects of the crosstalk between the gut microbiota and the intestinal epithelium. The publication was the first to demonstrate that gut microbes induce a specific iron-related protein signature. I performed some experiments, prepared figure n°5, wrote a small part of the manuscript (material/method & a section of the results) reviewed it and handled the revision.

14. Flt3 ligand improves the innate response to respiratory syncytial virus and limits lung disease upon RSV reexposure in neonate mice.

European journal of immunology, 2016, 46 (4), 874-884. DOI : 10.1002/eji.201545929

**Remot, A.**, Descamps, D., Jouneau, L., Laubreton, D., Dubuquoy, C., Bouet, S., Lecardonnel, J., Rebours, E., Petit Camurdan, A., Riffault, S. (2016).

Period: PhD

Contribution: I performed all experiments presented, analyzed the data, prepared the figures, and wrote the entire manuscript. I took care of the submissions and revisions (plurals, they were many! PLoSPathogens, Mucosal Immunology, Journal of Immunology, mBio... and European Journal of Immunology eventually) and correspondence with all co-authors. The manuscript was submitted to PLoSPathogens before my PhD's viva, and I took care of the follow-up listed below during my post-doctorate.

15. Visualizing the replication of respiratory syncytial virus in cells and in living mice.

Nature Communications, 2014, 5. DOI : 10.1038/ncomms6104

Rameix-Welti, M.-A., Le Goffic, R., Hervé, P.-L., Sourimant, J., **Remot, A.**, Riffault, S., Yu, Q., Galloux, M., Gault, E., Eléouët, J. F.

Period: PhD

Contribution: This article was written by virologists from my PhD's unit, who built recombinant RSV by reverse genetic. I tested different virus batches *in vivo* in adult BALB/c mice (intranasal infection and evaluation of viral load at several time points, by q-RT-PCR and bioluminescent imaging, 3 independent experiments). I did not realize the figures nor contributed to the writing.

16. Nucleoprotein nanostructures combined with adjuvants adapted to the neonatal immune context: a candidate mucosal RSV vaccine.

Plos One, 2012, 7 (5), DOI : 10.1371/journal.pone.0037722

**Remot, A.**, Roux, X., Dubuquoy, C., fix, J., Bouet, S., Moudjou, M., Eleouet, J. F., Riffault, S., Petit Camurdan, A.

Period: PhD

Contribution: I performed all experiments presented, analyzed the data, prepared the figures, wrote the materials/methods and legends of figures (the manuscript was written by Dr. A. Petit-Camurdan and Dr. S. Riffault). I reviewed the manuscript before submission and helped for the revision (experiments and text modifications).

17. Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells.

European Journal of Immunology, 2011, 41, 2852-2861. DOI : 10.1002/eji.201041224

**Remot, A.\***, Roux, X.\*, Petit Camurdan, A., Nahori, M.-A., Kiefer-Biasizzo, H., Marchal, G., Lagranderie, M., Riffault, S. \*co-first authors

Period: PhD

Contribution: this article was submitted as a short communication with 2 figures before my arrival in the laboratory. The requested revision was substantial and justified to change the type to a regular article. I realized all the required experiments (6 months of work), and prepared 3 new figures (Figures 2, 3 and 4). I re-wrote the article with the help of Dr. S. Riffault (redaction of materials/methods, legends of figures 2, 3, 4; and part of the results and discussion). I am co 1<sup>st</sup> author with the previous PhD student of the team.

18. Mesenteric lymph node cells from neonates present a prominent IL-12 response to CpG oligodeoxynucleotide via an IL-15 feedback loop of amplification.

Veterinary Research, 2011, 42, 19. DOI : 10.1186/1297-9716-42-19

Ferret-Bernard, S., Lacroix-Lamandé, S., **Remot, A.**, Metton, C., Bernardet, N., Charley, B., Drouet, F., Laurent, F.

Period: Master 2.

Contribution: Under the supervision of Dr. S. Ferret-Bernard and Dr. F. Laurent, I realized post-mortem organs sampling on lambs and ewes, and isolated cell types and labelling (for FACS cell sorting), I did *in vitro* stimulation experiments and ELISA. I also handled the bacteriology work presented on the 3rd figures. I did not prepare the figures nor contributed to the writing of the manuscript.

19. Cellular and molecular mechanisms underlying the strong neonatal IL-12 response of lamb mesenteric lymph node cells to R-848.

Plos One, 2010, 5 (10), e13705. DOI : 10.1371/journal.pone.0013705

Ferret-Bernard, S., **Remot, A.**, Lamandé, S., Metton, C., Bernardet, N., Drouet, F., Laurent, F.

Period: Master 2.

Contribution: Under the supervision of Dr. S. Ferret-Bernard and Dr. F. Laurent, I realized post-mortem organs sampling on lambs and ewes, and isolated cell types and labelling (for FACS cell sorting), I did *in vitro* stimulation experiments, ELISA, and prepared cell samples for transmission electron microscopy (TEM) and scanning electron microscopy (SEM). I did not prepare the figures nor contributed to the writing of the manuscript.

Summary of contributions to publications:

Position	Number of publications	Journal and year of publication
1 <sup>st</sup>	7	INRAE Prod. Animales (2022, in press), Frontiers in Vet. Science (2021) Frontiers Immunol (2019), ISME J (2017), Eur. J Immunol (2016 et 2011), PloSONE (2012)
Last	2 + 1 submitted	J. Dairy Science (submitted, available in BioRxiv), Frontiers Immunol (2021) Methods Mol Biol (2021)
2 <sup>nd</sup>	2	FASEB J (2016), PloSONE (2010)
Before last	1	Frontiers Physiol (2018)
Listed as author	7	FASEB J (2021), Journal of Immunology (2021), Vaccines (2021, 2020), Frontiers Immunol (2019), Nat Commun (2014), Vet Res (2011)
<a href="https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date">https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date</a> ORCID ID : <a href="https://orcid.org/0000-0001-9896-3216">0000-0001-9896-3216</a>		

## B. Patents

1 Patent Co-inventor (**A. Remot** 45%, M. Thomas 45%, P. Langella 10%), n°1650565, PCT/EP2017/051839: « Souche bactérienne comme agent de prévention et/ou agent de traitement des pathologies respiratoires » (bacterial strain as preventive or therapeutic agent against respiratory pathologies).

3 DIRV - invention Disclosures (inventor at 30%, 20% and 15%)

- DIRV (INRAE DI-RV-19-0102) « Identification d'une population de neutrophiles avec des propriétés immunorégulatrices chez la souris et le bovin » (identification of a neutrophil subset with immunomodulating properties in mice and cattle). Inventors: N. Winter (40%), **A. Remot (30%)** et E. Doz-Deblauwe (30%). Deposited October 2019.
- DIRV (INRAE/CNRS) « Utilisation de bactéries isolées du microbiote pulmonaire (et leur dérivés) pour la protection et le traitement d'infections respiratoires » (Use of lung bacteria -and their derivative products- to protect and treat lung infections). Inventors : G. Lugo (20%) L. Bernard (20%), O. Neyrolles (15%), M. Thomas (15%), P. Langella (15%), **A. Remot (15%)**. Deposited June 2018.
- DIRV (INRAE) « Utilisation de bactéries pulmonaires (ou de dérivés de bactéries pulmonaires) comme agents de prévention et/ou de traitement vis-à-vis de pathologies virales respiratoires du nouveau-né. » (Use of lung bacteria -and their derivative products- to protect and treat neonatal lung viral infections). Inventors: D. Descamps (25%), S. Riffault (25%), **A. Remot (25%)**, M. Thomas (25%). Deposited November 2017.

### C. Oral communications

Here after, 10 selected presentations and 7 as invited speaker are sorted by most recent.

**[Selected oral presentation – project awarded by a 10k€ FéRI price during the meeting]** Remot A., Eymieux S., Maquart M., Bull A., Carreras F., Sizaret D., Legras A., Winter N., Brand D. (2022-07-04). *MyCOVID project: Comparison of innate lung responses after ex vivo infection by mycobacteria from the M. tuberculosis complex and the SARSCoV-2*. **Presented at:** Journées d'Animation Scientifique de la FéRI 2022 - FéRI Scientific Days 2022, Joué-Lès-Tours, France (2022-07-04 - 2022-07-05), <https://hal.inrae.fr/hal-03735425>

**[Selected oral presentation]** Remot A., Carreras F., Doz E, Boschioli ML, Browne J, Gordon S, and Winter N. (2022-06-08). *Mycobacterial infection of precision cut lung slices reveals type 1 interferon pathway is locally induced by Mycobacterium bovis but not M. tuberculosis*. **Presented at:** 7th international conference on Mycobacterium bovis 2022, Galway, Ireland (2022-06-07 - 2022-06-10)

**[Selected oral presentation]** Remot A., Doz-Deblauwe E., Salle G., Le Vern Y., Sausset A., Winter N., Lacroix-Lamandé S. (2019-10-17). *Immune metabolism programming in bovine innate cells*. **Presented at :** Journées d'animation scientifique de la Fédération de recherche en Infectiologie (FéRI), Tours, France (2019-10-17), <https://hal.inrae.fr/hal-02935534>

**[Invited speaker]** Remot A., Doz-Deblauwe E., Winter N. (2018-12-13). *Caractérisations des populations de neutrophiles murins et bovins*. **Presented at:** Séminaire d'animation scientifique, unité VIM, Jouy-en-Josas, France (2018-12-13), <https://hal.inrae.fr/hal-02935376>

**[Invited speaker]** Remot A., Winter N. (2018-10-18). *Precision cut lung slices: an ex vivo model to monitor and evaluate innate tissue responses following Mycobacterium bovis infection*. **Presented at:** Modèles ex vivo, Journées d'animation scientifique département MICA, Paris, France (2018-10-18), <https://hal.inrae.fr/hal-02935367>

**[Selected oral presentation]** Remot A., Carreras F., Epardaud M., Doz E., Archer F., Boschioli M. L., Biet F., Winter N. (2018-03-26). *Precision-cut lung slices: an ex vivo model to monitor and evaluate innate tissue responses following Mycobacterium bovis infection*. **Presented at:** 11. Symposium of the French Domestic Animal Immunology Network (IAD 2018), Tours, France (2018-03-26 - 2018-03-27), <https://hal.inrae.fr/hal-02736323>

**[Invited speaker]** Remot A., Winter N., Biet F. (2017-11-23). *Intergrative research on pathogenic mycobacteria at INRA*. **Presented at:** Tuberculosis as a One Health Paradigm: Opportunities for Consortium Building and EU Framework Programme Funding, Dublin, Irlande (2017-11-23 - 2017-11-24), <https://hal.inrae.fr/hal-02790408>

**[Invited speaker - Keynote lecture]** Aude Remot (2016-04-13). Lung bacterial imprinting in allergic asthma susceptibility. **Presented at:** 6th Journées des doctorants BSB, Toulouse, France, (2016-04-13), <https://hal.inrae.fr/hal-02738670>.

**[Invited speaker]** Remot, A., Thomas, M. (2016-02-11). Des modèles expérimentaux à la clinique : Contribution du microbiote pulmonaire dans l'asthme. **Presented at** 3rd Journée d'Allergologie de l'Est Parisien de l'enfant à l'adulte (JAEP), Paris, France (2016-02-11 - 2016-02-11). <http://prodinra.inra.fr/record/368674>

**[Selected oral presentation]** Remot, A., Noordine, M.-L., Bridonneau, C., Descamps, D., Riffault, S., Langella, P., Thomas, M. (2015-06-18). Impact of the development of the lung microbiota on neonatal immunity. **Presented at** 20. Colloque du Club des Bactéries Lactiques, Lille, France (2015-06-17 - 2015-06-19). <http://prodinra.inra.fr/record/368706>

**[Invited speaker]** Remot A. (2015-11-17). *Flt3 ligand improves innate responses to respiratory syncytial virus in neonate mice and limits lung disease upon virus re-exposure*. **Presented at:** Séminaire Hôpital Saint Antoine, Paris, France (2015-11-17 - 2015-11-17). <https://hal.inrae.fr/hal-02792503>

**[Selected oral presentation - Best presentation award]** Remot A., Descamps D, Jouneau L, Dubuquoy C, Bouet S, Lecardonnel J, Rebours D, Cherbuy C, Petit-Camurdan A, Riffault S (2014-09-14). Lung neonatal responses to respiratory syncytial virus reveal deficiencies in dendritic cells and type I interferon in a mouse model of airway enhanced disease. **Presented at:** 13th International Symposium on Dendritic Cells, Tours, France (2014-09-14), <https://hal.inrae.fr/hal-02739158>

**[Selected oral presentation - Best presentation award]** Remot, A., Jouneau, L., Descamps, D., Dubuquoy, C., Bouet, S., Lecardonnel, J., Rebours, E., Cherbuy, C., Petit-Camurdan, A., Riffault, S. (2014). Lung neonatal responses to respiratory syncytial virus reveal deficiencies in dendritic cells and type I interferon in a mouse model of airway enhanced disease. **Presented at** 8th World Immune Regulation Meeting, Davos, Swiss (2014-03-19 - 2014-03-22). <http://prodinra.inra.fr/record/368738>

**[Selected oral presentation]** Remot A., Thomas M., Hammad H. (2013-11-04). *Impact of lung microbiota on neonatal immunity*. **Presented at:** 1st AgreenSkills Annual Meeting 2013, Leuven, Belgique (2013-11-04 - 2013-11-06), <https://hal.inrae.fr/hal-02808501>

**[Selected oral presentation]** Remot, A., Jouneau, L., Descamps, D., Dubuquoy, C., Bouet, S., Lecardonnel, J., Rebours, E., Cherbuy, C., Petit-Camurdan, A., Riffault, S. (2012-12-13). Transcriptomic analysis of neonatal vs. adult lung early responses to RSV reveals key roles for DC and type I IFN in controlling immunopathologic imprinting., **Presented at** : Diversity and plasticity of DCs SFI meeting, Paris, France, (2012-12-13), <https://hal.inrae.fr/hal-01191380>

**[Selected oral presentation]** Remot A., Petit Camurdan A., Dubuquoy C., Bernard J., Moudjou M., Eleouet J. F., Riffault S. (2011-05-22). *Newborns are particularly prone to respiratory syncytial virus infection: why? How to protect them?*. **Presented at:** Journées d'Animation

Scientifique du Département Santé Animale, Fréjus, France (2011-05-22 - 2011-05-25), <https://hal.inrae.fr/hal-02744806>

[**Invited speaker**] Remot A., Riffault S. (2010-09-23). *Which immune lung features relate to the higher severity of respiratory syncytial virus infection in newborns?* Presented at: Séminaire, Institute for Virology, University of veterinary medicine, Hanover, Germany (2010-09-23 - 2010-09-23) <https://hal.inrae.fr/hal-02813237>

#### D. Posters

20 posters sorted by most recent here after.

The name of the person who presented the poster is underlined. OA: Open access on HAL website.

[1 – **best poster award**] Rambault M., Doz-Deblauwe E., Le Vern Y., Carreras F., Cunha P., Germon P., Rainard P., Winter N., Remot A. (2021-08-29). *Neutrophils encompass a regulatory subset suppressing T cells at steady state in cattle.* Presented at: 7th European Veterinary Immunology Workshop (EVIW), Online, (2021-08-29 - 2021-08-31), <https://hal.inrae.fr/hal-03382738>, OA

[2] Remot A., Carreras F., Doz-Deblauwe E., Boschioli M. L., Biet F., Gordon S. V., Winter N. (2020-12-17). *The study of bovine tuberculosis through a One Health approach.* Presented at: VetBioNet annual meeting (2020-12-17 - 2020-12-17)

[3] Remot A., Carreras F., Doz-Deblauwe E., Boschioli M. L., Biet F., Gordon S. V., Winter N. (2019-10-17). *The study of bovine tuberculosis through a One Health approach.* Presented at: Journées d'animation scientifique de la Fédération de Recherche en Infectiologie (FÉRI), Tours, France (2019-10-17), <https://hal.inrae.fr/hal-02935282>

[4] Remot A., Carreras F., Epardaud M., Doz-Deblauwe E., Archer F., Boschioli M. L., Biet F., Winter N. (2018-10-08). *Precision-cut lung slices: an ex vivo model to monitor and evaluate innate tissue responses following Mycobacterium bovis infection.* Presented at: Journées d'Animation Scientifique du Département Santé Animale, Nantes, France (2018-10-08 - 2018-10-11), <https://hal.inrae.fr/hal-02738077>

[5] Vinai T., Doz E., Carreras F., Biet F., Boschioli M. L., Winter N., Remot A. (2018-03-26). *Bovine neutrophils characterization and interactions with Mycobacterium bovis.* Presented at: 11. Symposium of the French Domestic Animal Immunology Network (IAD 2018), Tours, France (2018-03-26 - 2018-03-27), <https://hal.inrae.fr/hal-02737457>

[6] Vinai T., Doz-Deblauwe E., Carreras F., Biet F., Boschioli M. L., Winter N., Remot A. (2018-10-08). *Bovine neutrophils characterization and interactions with Mycobacterium bovis.* Presented at: Journées d'Animation Scientifique du Département Santé Animale, Nantes, France (2018-10-08 - 2018-10-11), <https://hal.inrae.fr/hal-02737322>



- [7] Carreras F., Doz E., Epardaud M., Remot A., Cochard T., Biet F., Winter N. (2017-05-29). *Obtention de souches fluorescentes de Mycobacterium bovis*. Presented at : 5th Conférence du MycoClub (Mycobactéries 2017), Montigny-le-Bretonneux, France (2017-05-29 - 2017-05-31), <https://hal.inrae.fr/hal-02734098>, OA
- [8] Remot A., Epardaud M., Carreras F., Tomal F., Doz E., Biet F., Winter N. (2017-05-29). *Crosstalk between bovine alveolar macrophages and Mycobacterium bovis*. Presented at: 5th Conférence du MycoClub (Mycobactéries 2017), Montigny-le-Bretonneux, France (2017-05-29 - 2017-05-31), <https://hal.inrae.fr/hal-02737456>
- [9] Remot A., Epardaud M., Biet F., Winter N. (2016-03-17). *Bovine innate immunity against Mycobacterium bovis*. Presented at: IAD 2016 - 10th Journées du Réseau Français "Immunologie des animaux domestiques", Ploufragan, France (2016-03-17 - 2016-03-18), <https://hal.inrae.fr/hal-02740245>
- [10] Remot A., Descamps D., Jouneau L., Dubuquoy C., Bouet S., Lecardonnel J., Rebours E., Cherbuy C., Petit-Camurdan A., Riffault S. (2014-09-14). *Lung neonatal responses to respiratory syncytial virus reveal deficiencies in dendritic cells and type I interferon in a mouse model of airway enhanced disease*. Presented at: 13th International Symposium on Dendritic Cells, Tours, France (2014-09-14 - 2014-09-18), <https://hal.inrae.fr/hal-02739158>, OA
- [11] Remot A., Penel A., Bauducel M., Bridonneau C., Descamps D., Riffault S., Langella P., Hammad H., Thomas M. (2014-03-19). *Impact of the development of the pulmonary microbiota on neonatal immunity*. Presented at: 8th World Immune Regulation Meeting, Davos, Suisse (2014-03-19 - 2014-03-22), <https://hal.inrae.fr/hal-02742629>, OA
- [12] Remot A., Penel A., Bauducel M., Bridonneau C., Descamps D., Riffault S., Langella P., Hammad H., Thomas M. (2013-11-04). *Impact of the development of the pulmonary microbiota on neonatal immunity*. Presented at: AgreenSkills Annual Meeting 2013, Louvain, Belgique (2013-11-04 - 2013-11-06), <https://hal.inrae.fr/hal-02748060>
- [13] Remot A., Jouneau L., Dubuquoy C., Bouet S., Lecardonnel J., Rebours E., Cherbuy C., Petit Camurdan A., Riffault S. (2012-12-13). *Transcriptomic analysis of neonatal vs. adult lung early responses to RSV reveals key roles for DC and type I IFN in controlling immunopathologic imprinting*. Presented at: Diversity and plasticity of DCs, Club Francophone des Cellules Dendritiques, Paris, France (2012-12-13), <https://hal.inrae.fr/hal-02935313>
- [14] Remot A., Djavidi A, Dubuquoy C, Schwartz-Cornil I, Laurent F, Morel O, Chavatte Palmer P, Perez N, Petit Camurdan A, Riffault S (2012-09-02). *Age-related differences in the lung tissue susceptibility to Respiratory Syncytial Virus*. Presented at: 4th European Veterinary Immunology Workshop (EVIW), gb (2012-09-02), <https://hal.inrae.fr/hal-02748020>

[15] Aude Remot, Roux X, Dubuquoy C, Bernard J, Fix J, Moudjou M, Eleouet J.F, Petit Camurdan A, Riffault S (2011-11-16). *Nucleoprotein nanostructures: a mucosal subunit vaccine candidate for neonates protective against the respiratory syncytial virus*. Presented at: Cold spring Harbor meeting: Harnessing immunity to treat and prevent diseases, us (2011-11-16), <https://hal.inrae.fr/hal-02745116>

[16] Aude Remot, Roux X, Dubuquoy C, Bernard J Moudjou M, Eleouet J.F, Riffault S, Petit Camurdan A (2011-07-05). *Neonatal Nasal Vaccination with the Nucleoprotein of the Respiratory Syncytial Virus Elicits Virus-protective but Airway-pathogenic Th2-biased Immunity that can be Modulated by the Choice of Adjuvants*. Presented at: 15th International Congress of Mucosal Immunology (ICMI 2011) "Peace and War at Mucosal Surfaces", fr (2011-07-05), <https://hal.inrae.fr/hal-02745248>

[17 – **Best poster award**] Remot A., Roux X., Dubuquoy C., Bernard Theron J., Fix J., Moudjou M., Eleouet J. F., Petit-Camurdan A., Riffault S. (2011-03-29). *Nucleoprotein nanostructures: a mucosal subunit vaccine candidate for neonates protective against the respiratory syncytial virus*. Presented at: Journées de l'Ecole doctorale ABIES, Paris, France (2011-03-29 - 2011-03-30), <https://hal.inrae.fr/hal-02805147>

[18] Remot A., Djavid A., Petit-Camurdan A., Dubuquoy C., Schwartz-Cornil I., Laurent F., Morel O., Chavatte-Palmer P., Parez N., Riffault S. (2010-05-10). *Rôle des récepteurs PAR et TLR pulmonaires dans les réponses néonatales aux infections virales respiratoires*. Presented at: 6th Colloque DOC'J 2010 "La biologie et les sciences qui gravitent autour d'elle", Jouy-en-Josas, France (2010-05-10 - 2010-05-11), <https://hal.inrae.fr/hal-02754675>, OA

[19] Remot A, Ferret-Bernard S, Suleman M, Galea S, Foucras G, Schwartz-Cornil I, Richardson J, Laurent F (2009-09-10). Metabolically biotinylated adenovirus for ovine dendritic cell targeting and ligand screening. 1 p., Presented at: 3d EVIW European Veterinary Immunology Workshop, de, (2009-09-10), <https://hal.inrae.fr/hal-01409475>

[20] Ferret-Bernard S, Remot A, Bernardet B, Lamandé S, Laurent F (2009-09-10). CD14+ cells contribute to the higher level of IL-12 secretion in response to TLR ligands by neonatal lamb cells compared to adult cells. 1 p., Presented at: 3d EVIW European Veterinary Immunology Workshop (EVIW), de, (2009-09-10), <https://hal.inrae.fr/hal-01409473>

### E. Scientific communication to the general public

I use my results to feed outreach activities locally, such as the **French Science Festival** (annual event at Tours city hall). Each year since 2016, I participate to the brainstorming meetings to decide the communication supports and, together with my colleague Emilie Doz-Deblauwe, I help for the realization of those related to immunology: Poster about the One Health concept in the ISP unit; bovine cells and pathogens spotted for observation under light microscopes; cartoons to explain the different immune cells etc. During the Science Festival, I spend half a day on the ISP stand, to explain our research and guide children -and sometimes adults!-

through some games: pipetting mint sirop to make a good standard curves; recognized neutrophils and bacteria under a microscope; create your own bacteria or immune cells with tissue paper and fancy accessories etc...

In June this year, I also initiated children to microbes (the good and bad guys) and the immune system, our army of soldiers present to defend our body. I spent 1h per class in a **primary school and a kindergarten** for a total of 10h. With the youngest, I did easy Science experiments with the help and assistance of my PhD Marion Rambault: pepper and soap; hands in Petri dishes and observation of bacterial colonies the next day; fluorescent gel and a UV light box to evaluate hands washing etc... With the oldest, we exchanged about microbes (which and where they are), our immune system and vaccination. This initiation was part of an event organized by the French Society of Immunology to promote scientific curiosity at school (Immunology ambassador for kids).

During my PhD, I participated to the **Paris International Agricultural Show** (in 2011), to promote the importance of animal health. I created some communication supports with the help of the INRAE team (cartoons and posters), and was present during one day at the stand to explain our research, talk and play with every generations: evaluate the size of virus, bacteria, cells with common visual support (a house, a tree, a human, a mice, a rock etc...), learn how to dress correctly to work in a bio contained safety laboratory (glasses, hear and shoes covers, gloves, Tyvek... what to put first and why), observe fluorescent cells under a fluorescent microscopes (it was fish cells transfected with a GFP-plasmid).

My ANR JCJC Neutro\_bTB project long-term impact will **profit to both animal health and agriculture economy**. The main beneficiaries being people from the cattle sector, my future results will be **disseminated during breeders and practitioners' meetings in France** (3R meeting: "Rencontre autour de la Recherche sur les Ruminants") and published as non-scientific articles in journals and newsletters targeting these actors (published by the French Institut de l'élevage for instance).

To reach the general audience and promote scientific culture, I broadcast some of my main findings, in a way adapted to non-scientific readers, via my Twitter account. I also advertise my project and positions (Master2 internship mostly) via **twitter, LinkedIn and ResearchGate**.

# Neonatal lung immune responses show a shift of cytokines and transcription factors toward Th2 and a deficit in conventional and plasmacytoid dendritic cells

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Marie-Anne Nahori<sup>2</sup>, Hélène Kiefer-Biasizzo<sup>2</sup>, Gilles Marchal<sup>2</sup>,  
Micheline Lagranderie<sup>2</sup> and Sabine Riffault<sup>1</sup>

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The high incidence of lung-damaging life-threatening respiratory infections in infants may be related to the immaturity of their immune systems. To determine whether lung immune features differ in early life compared with those in adulthood, whole lung as well as lung T lymphocyte and DC responses were investigated in BALB/c neonates versus adults. Higher expression of GATA-3 and rapid and sustained production of type 2 cytokines by lung explants after *in vitro* exposure to anti-CD3 was the hallmark of the neonatal period, suggestive of a Th2 bias. Neonatal lung GATA-3-producing cells were identified as CD3<sup>+</sup>, CD4 and CD8 double-negative T lymphocytes, a subset found at a higher frequency in neonatal than adult lung. The neonatal lungs contained fewer conventional DCs, with a lower ratio of CD103<sup>+</sup> to CD11b<sup>+</sup> DCs, and a much lower number of plasmacytoid DCs in comparison with adult lungs. Yet, when stimulated *in vivo* by BCG, neonatal lung DCs matured and primed adult naïve CD4<sup>+</sup> T cells toward Th1 as efficiently as adult BCG-primed lung DCs. Conversely, both adult and neonatal BCG-primed lung DCs induced a Th2 cytokine response from neonatal naïve lymph node T cells, suggestive of an intrinsic feature of neonatal T lymphocytes.

**Key words:** DCs · Lung · Neonates · Th2



Supporting Information available online

## Introduction

Childhood, noticeably the neonatal period, is marked by a high susceptibility to infectious diseases. About 20% of all deaths in children under 5 years of age are due to acute lower respiratory infections (pneumonia, bronchiolitis and bronchitis) caused by bacteria (most commonly *Streptococcus pneumoniae* and *Haemo-*

*philus influenzae*) or viruses (most commonly respiratory syncytial virus (RSV)) [1].

The higher susceptibility to infectious diseases has been attributed to the so-called “immaturity” of the neonatal immune system. The characteristics of neonatal Th responses have been well documented in one-wk old BALB/c mice. BALB/c neonates commonly demonstrate enhanced Th2 functions at all phases of the immune response and poor Th1 memory responses resulting in an overall Th2-dominance during early life [2]. This Th2 immune profile was shown to be an intrinsic feature of the CD4<sup>+</sup> T cells within the secondary lymphoid organs, which, when

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extracted from their neonatal environment and transferred into adult recipients, produce high levels of the type 2 cytokine IL-4 and are deficient for the production of the type 1 cytokine IFN- $\gamma$  [3]. Two mechanisms may contribute to the neonatal Th2 bias. First, the Th2 cytokine locus is hypomethylated during the neonatal period, thus allowing rapid high-level Th2 gene transcription (*IL-4*, *IL-5* and *IL-13*) [4]. Second, neonatal Th1 cells express high levels of interleukin 13 receptor  $\alpha 1$  (IL-13R $\alpha 1$ ), making them prone to apoptosis driven by IL-4, which is produced by activated Th2 cells [5]. Furthermore, BALB/c neonates lack the splenic CD8 $\alpha^+$ CD4 $^-$  DC subset that is required for IL-12-mediated downregulation of IL-13R $\alpha 1$ , allowing Th1 memory to arise [5]. It is nevertheless possible to generate adult-like immune responses during the neonatal period, if the appropriate stimuli and cellular partners are gathered in the right settings [6]. For instance, CpG oligodeoxynucleotides added to protein or live vaccine can circumvent the neonatal Th2 polarization [7].

Our present study is focused on the neonatal immune features of the lung, a major site of encounter between respiratory pathogens and the mucosal immune system. We explored the capacity of lung T lymphocytes to produce type 1 or type 2 cytokines upon polydonal CD3 activation of lung explants, and the expression in lung cell suspensions of GATA-3 and T-bet, the transcription factors indicative of Th2 and Th1 programming respectively [8].

We next compared DC subsets in neonatal versus adult lungs, as early studies showed that the respiratory-tract intraepithelial network of MHC class II (MHCII)-bearing DCs is severely impaired during the neonatal period until after weaning in the rat [9]. Both conventional DC (cDC) and plasmacytoid DC (pDC) subsets have now been identified in lungs of adult mice and ascribed with roles in the pathogenesis of Th2-mediated lung diseases [10, 11]. The CD103 $^+$  cDC subset found in peripheral mucosal tissues is developmentally and functionally related to the CD8 $\alpha^+$  cDC subset, known to be deficient in spleen and lymph nodes during the neonatal period [12]. We investigated whether these different DC subsets were impaired in neonatal lungs and whether monocytic DC precursors were present [13]. Finally, since we had previously shown that BCG delivered intranasally (i.n.) to adult BALB/c mice is taken up by lung DCs that can then prime naive T cells in vitro for a long-lasting Th1 response [14], we adopted the same experimental settings to investigate the capacity of neonatal lung cDCs to mature and prime naive CD4 $^+$  T cells, isolated from lymph nodes of mice at various ages.

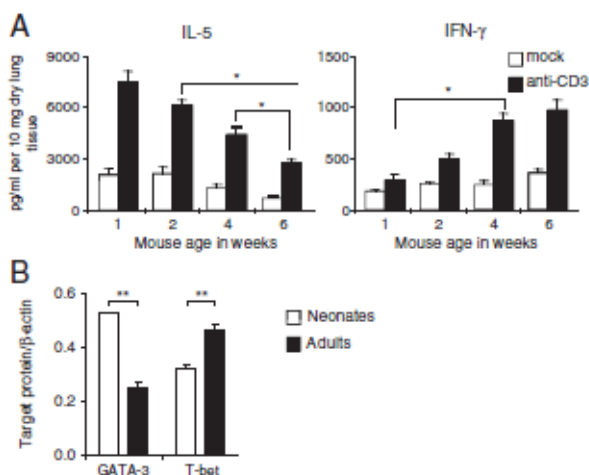
We observed that the neonatal lung T lymphocytes were prone to produce type 2 cytokines and over-express the Th2-associated GATA-3 transcription factor, the latter being mainly found within a subset of CD4/CD8 double-negative CD3 $^+$  lymphocytes over-represented in neonatal lungs. Both pDCs and cDCs were found numerically deficient in neonatal lungs with a more pronounced deficit in the CD103 $^+$  cDC subset. Yet, after in vivo stimulation with BCG, neonatal lung DCs had the same capacity as adult lung DCs to mature and prime naive adult lymph node CD4 $^+$  T cells for Th1 cytokine production, whereas

neonatal naive CD4 $^+$  T cells produced Th2 cytokines, even if cultured with BCG-activated adult lung DCs.

## Results

### Lung T cells display a distinct type 2 profile during the neonatal period

To explore whether T cells resident in neonatal lungs have biased features, we investigated the whole lung ability to secrete prototypic type 1 (IFN- $\gamma$ ) and type 2 (IL-4, IL-5) cytokines upon CD3 polyclonal activation. Lungs were collected from naive mice at various ages (from 1 to 6 wk) and lung explants were further incubated with or without soluble anti-CD3 mAb. Neonatal lung explants secreted more IL-5 than their adult counterparts, and the amount of secreted IL-5 decreased with age, with and without anti-CD3 stimulation (Fig. 1A). Similar observations were recorded with IL-4 (data not shown). Conversely, the IFN- $\gamma$  secretion was low in neonates and increased with age, especially upon stimulation with anti-CD3 mAb (Fig. 1A). We next investigated whether the neonatal lung tissue showed a Th2-biased transcription factor profile. The levels of expression of GATA-3 and T-bet were monitored by Western blot analysis in total lung protein extracts obtained from neonates or adults. The expression of GATA-3 was stronger and conversely the level of T-bet was lower in neonates than in adults (Fig. 1B).



**Figure 1.** Biased Th2 responses in neonatal lungs. (A) Lung explants of 1-, 2-, 4- and 6-wk-old BALB/c mice were cultured for 6 h (IL-5) or 24 h (IFN- $\gamma$ ) with or without addition of anti-CD3 mAb. The secretion of IL-5 and IFN- $\gamma$  was measured in supernatants by Bio-Plex cytokine assay. (B) The level of expression of the transcription factors GATA-3 and T-bet was determined by Western blot in protein extracts prepared from total lung. Data were acquired and normalized to  $\beta$ -actin using a ScanAnalyze. Data are mean  $\pm$  SEM from  $n = 3$  mice and repeated twice. Statistical analysis was carried out using the Student's  $t$ -test (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

To assess whether the differential GATA-3 and T-bet expression between neonatal and adult lungs could be related to different proportions of lung T lymphocyte subsets, we analyzed in lung cell suspensions from neonate and adult BALB/c mice the main CD3<sup>+</sup> T-cell subsets (CD4<sup>+</sup>, CD8<sup>+</sup>, DN CD4<sup>−</sup>CD8<sup>−</sup>, DP CD4<sup>+</sup>CD8<sup>+</sup>, NKT DX5<sup>+</sup> and TCR- $\gamma\delta$ <sup>+</sup>). Compared with adults, BALB/c neonates had fewer lung cells ( $9.6 \pm 0.5$  versus  $13.8 \pm 0.9$  million cells,  $n = 20$ ,  $p < 0.001$ ) and a percentage of CD3<sup>+</sup> T cells nearly three-fold lower ( $4.1 \pm 0.4\%$  versus  $11.4 \pm 2.9\%$ ,  $n = 4$ ,  $p < 0.05$ , Fig. 2A).

Among lung CD3<sup>+</sup> T cells, the CD4<sup>+</sup> and CD8<sup>+</sup> subsets were significantly reduced and conversely the DN CD4<sup>−</sup>CD8<sup>−</sup> subset was significantly enriched in neonates compared with adults, while the DP CD4<sup>+</sup>CD8<sup>+</sup> subset was scarce in both adults and neonates (Fig. 2A). There were no significant differences in the proportion of NKT (CD3<sup>+</sup>DX5<sup>+</sup>) and TCR- $\gamma\delta$ <sup>+</sup> T-cell subsets in neonatal and adult lungs, both being found at low frequency (Fig. 2A).

Next, GATA-3 and T-bet producing CD3<sup>+</sup> T cells were identified by intracellular staining in lung cells isolated from neonatal and adult mice. The CD3<sup>+</sup> gating strategy and isotype control stainings are depicted in Supporting Information Fig. 1.

Overall, neonatal lung CD3<sup>+</sup> T cells contained a significantly higher frequency of GATA-3<sup>+</sup> cells than their adult counterparts (Fig. 2B). Among neonatal CD3<sup>+</sup> T cells, the main subset expressing GATA-3 was the DN subset (accounting for nearly 80% of GATA-3<sup>+</sup> T cells), then the DX5<sup>+</sup> and CD4<sup>+</sup> subsets (accounting for respectively 12 and 8% of GATA-3<sup>+</sup> T cells). By contrast, the main lung T-cell subset producing GATA-3 in adult was the CD4<sup>+</sup> subset (accounting for nearly 50% of GATA-3<sup>+</sup> T cells) and then the DN and DX5<sup>+</sup> subsets (accounting respectively for 28 and 18% of GATA-3<sup>+</sup> T cells) (Fig. 2B).

Less than 1% of CD3<sup>+</sup> lung T cells were found to express T-bet, a proportion not significantly different between neonatal and adult lungs, with T-bet<sup>+</sup> CD3<sup>+</sup> T cells being mainly CD4<sup>+</sup> in neonates and CD4<sup>+</sup> or CD8<sup>+</sup> in adults (Fig. 2C).

We calculated the total number of T-bet<sup>+</sup>CD3<sup>+</sup> and GATA-3<sup>+</sup> CD3<sup>+</sup> cells in neonate and adult lungs and found significantly less T-bet<sup>+</sup>CD3<sup>+</sup> cells together with a higher ratio of GATA-3<sup>+</sup> over T-bet<sup>+</sup> T cells in neonate than in adult ( $n = 4$ ,  $p < 0.05$ , Fig. 2D).

Taken together, these results support our hypothesis that lung T lymphocytes have particular type 2 features during the neonatal period and pointed to the role of an original DN T-cell subset within neonatal lungs. We next explored in the lungs what DC subsets were present during the neonatal period.

### Lung cDC and pDC subsets are less abundant in neonates than in adults

The two main DC subsets, cDC and pDC, were investigated in lungs from neonatal (5–6 days old) and adult (6–8 wk old) BALB/c mice. First, lung cDCs were defined as CD11c<sup>+</sup>/MHCII<sup>high</sup> in neonatal and adult mice (Fig. 3A). According to our data (Fig. 3A), neonates had significantly less cDCs in their lungs, both in

percentage and number (1.7-fold less % cDCs and 3.3-fold less cDCs than adults). We next analyzed the two major lung cDC subsets (CD103<sup>+</sup> and CD11b<sup>+</sup>, described in [15]) based on the dual staining with anti-CD11b and anti-CD103mAb (Supporting Information Fig. 2A). The percentage of CD103<sup>−</sup>CD11b<sup>+</sup> cDCs (CD11b<sup>+</sup>) was significantly higher in neonates than in adults, whereas the percentage of CD103<sup>+</sup>CD11b<sup>−</sup> cDCs (CD103<sup>+</sup>) was significantly lower in neonates than in adults ( $n = 8$ ,  $p < 0.001$ ) (Fig. 3A). Accordingly, the ratio of CD103<sup>+</sup> to CD11b<sup>+</sup> lung cDCs was significantly lower in neonates than in adults ( $0.32 \pm 0.01$  for neonates versus  $0.68 \pm 0.05$  for adults,  $n = 8$ ,  $p < 0.001$ ) (Fig. 3A).

Second, we compared the number of pDCs in neonatal and adult lungs. We tested various phenotypic markers for pDCs (SiglecH, mPDCA1, B220) in combination with CD11b, CD11c and MHCII. For lung tissue, we found that mPDCA1 was the best marker to identify pDCs (the following results hold true when using the other pDC markers, data not shown). Our gating strategy and isotype control stainings are depicted in Supporting Information Fig. 2B. Although well represented in the spleen of neonates (Fig. 3B), pDCs were scarce in neonatal lungs and significantly less represented than in adult lungs both in percentage and number (2.7-fold lower % pDCs and 4.2-fold lower pDC number than adults) (Fig. 3B).

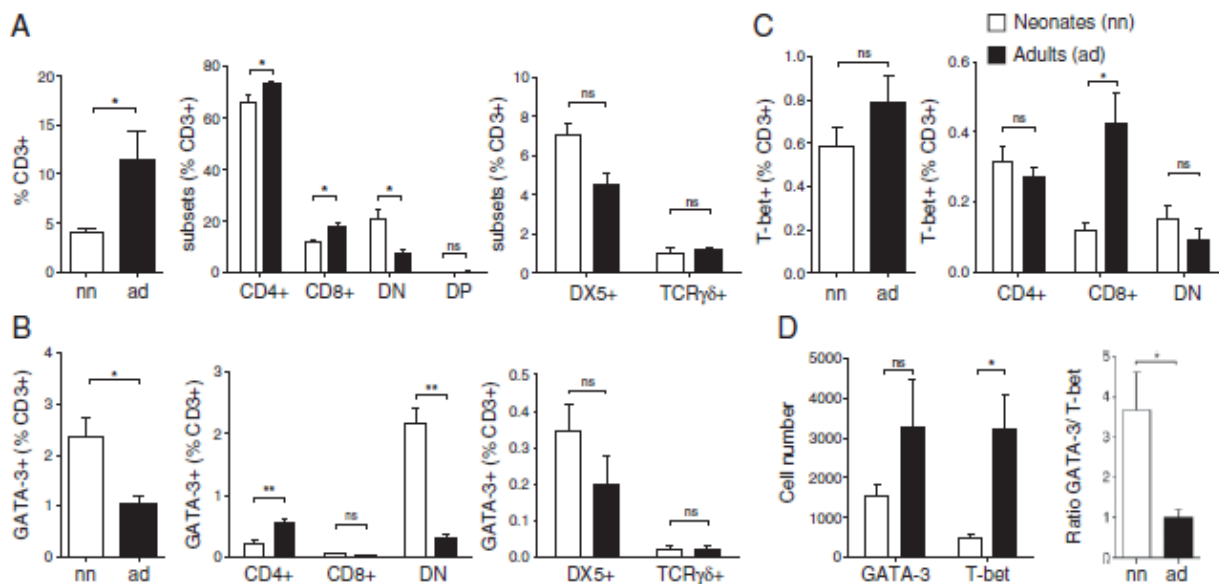
To achieve a complete overview of BALB/c neonatal lung DCs, we monitored the prevalence of their monocytic precursors by a dual Ly6c and CD11b staining, according to the previously published data [13]. We found that MHCII<sup>high</sup>CD11c<sup>+</sup>Ly6c<sup>+</sup>CD11b<sup>+</sup> DCs (Fig. 4, gate R1) were significantly less frequent in neonatal than in adult lungs (same tendency as depicted in Fig. 3A for MHCII<sup>high</sup>CD11c<sup>+</sup>cDC), whereas the MHCII<sup>low</sup>CD11c<sup>+</sup>Ly6c<sup>+</sup>CD11b<sup>+</sup> monocytic DC precursors (Fig. 4, gate R2) were more frequent in neonatal than adult lungs ( $25.5 \pm 2.2\%$  in neonates versus  $14.7 \pm 1.0\%$  in adults,  $n = 4$ ,  $p < 0.05$ ). Thus neonate lungs contained a large pool of monocytic DC precursors.

Finally, to get a hint of the neonatal lung DC ability to migrate, we monitored the expression of CCR7, the chemokine receptor for DC migration to draining LNs [16]. We found significantly more CCR7 expressed by neonatal lung cDCs (CD11b<sup>−</sup> and CD11b<sup>+</sup>) than adult (Supporting Information Fig. 4,  $n = 4$ ,  $p < 0.01$ ), suggesting their potent ability to migrate to draining lymph node.

### Neonatal lung cDCs primed in vivo with BCG or LPS are potent APCs

We finally investigated whether the neonatal lung cDCs have the capacities of functional APCs at steady state and after in vivo stimulation with strong inflammatory signals such as LPS or BCG. The capacity of DCs to phagocytose mycobacteria is well-documented in vivo [14, 17], however, the phagocytic capacity of neonatal lung DCs remained to be explored. Fluorescent BCG was administered i.n. to neonatal and adult mice, and CD11c<sup>+</sup>MHCII<sup>high</sup> lung cDCs were recovered at various time points. Though barely detectable in lung cDCs isolated from adults or





**Figure 2.** Neonate lungs are characterized by a major CD4<sup>+</sup>CD8<sup>-</sup> subset of T cells expressing GATA-3. Lung cells, isolated from neonatal or adult BALB/c mice, were surface labeled with anti-CD3-PerCP, anti-CD4-FITC and anti-CD8-biotin or -DX5-biotin or -TCR- $\gamma\delta$ -biotin followed by streptavidin- $\alpha$ -allophycocyanin, before intracellular staining with anti-GATA-3-PE or anti-T-bet-PE. At least 500 000 events were acquired on a FACS Calibur (gating strategy and isotype control staining are shown in Supporting Information Fig. 1). All data are mean  $\pm$  SEM, with  $n = 4$  pools of two mice. Data are representative of two independent experiments. Statistical analysis was carried out using Mann-Whitney U test (\* $p < 0.05$ ; \*\* $p < 0.01$ ). (A) The percentage of CD3<sup>+</sup> lymphocytes was determined among neonatal and adult lung cells. The percentages of single positive CD4<sup>+</sup>, CD8<sup>+</sup>, double-positive CD4<sup>+</sup>CD8<sup>+</sup> (DP), double-negative CD4<sup>-</sup>CD8<sup>-</sup> (DN), DX5<sup>+</sup> and TCR- $\gamma\delta$ <sup>+</sup> subsets were determined within the CD3<sup>+</sup> gate. The percentages of (B) GATA-3 or (C) T-bet expressing lung cells among the CD3<sup>+</sup> cells was determined for each subset (single positive CD4<sup>+</sup>, CD8<sup>+</sup>, DN CD4<sup>-</sup>CD8<sup>-</sup>, DX5<sup>+</sup> and TCR- $\gamma\delta$ <sup>+</sup>) and depicted as histograms for adults (black bars) and neonates (white bars). (D) The total number of GATA-3<sup>+</sup>CD3<sup>+</sup> or T-bet<sup>+</sup>CD3<sup>+</sup> cells per lung was calculated based on percentages determined by flow cytometry analysis (shown in B and C) and numeration of lung cells. Ratio of GATA-3<sup>+</sup>CD3<sup>+</sup> over T-bet<sup>+</sup>CD3<sup>+</sup> cells was calculated for each sample of neonatal lung (white bar) or adult lung (black bar).

neonates 1 h post-BCG delivery, 10 to 20 % of cDCs collected at later time points (6 h to 48 h post-BCG) appeared loaded with fluorescent BCG with no significant differences in proportion between neonates and adults (Fig. 5A).

CD11c<sup>+</sup>MHCII<sup>high</sup> lung cDCs isolated from untreated neonates or adults produced a very low amount of IL-12p40 (Fig. 5B). By contrast, neonatal or adult lung cDCs purified 48 h after i.n. delivery of BCG or LPS and cultured for 24 h in absence of any other stimuli produced similar large amounts of IL-12p40 (Fig. 5B). We did not detect IL-12p70 in the same supernatants (data not shown). Besides lung cDCs isolated from neonatal and adult mice 24 h after i.n. delivery of BCG or LPS expressed the co-stimulatory molecules CD80 and CD86 at similar high levels (Fig. 5C), while both were not detected above background staining in the non-treated counterparts (data not shown). Thus neonatal and adult lung cDCs displayed similar features of maturation (CD80, CD86, IL-12p40) after i.n. delivery of BCG or LPS.

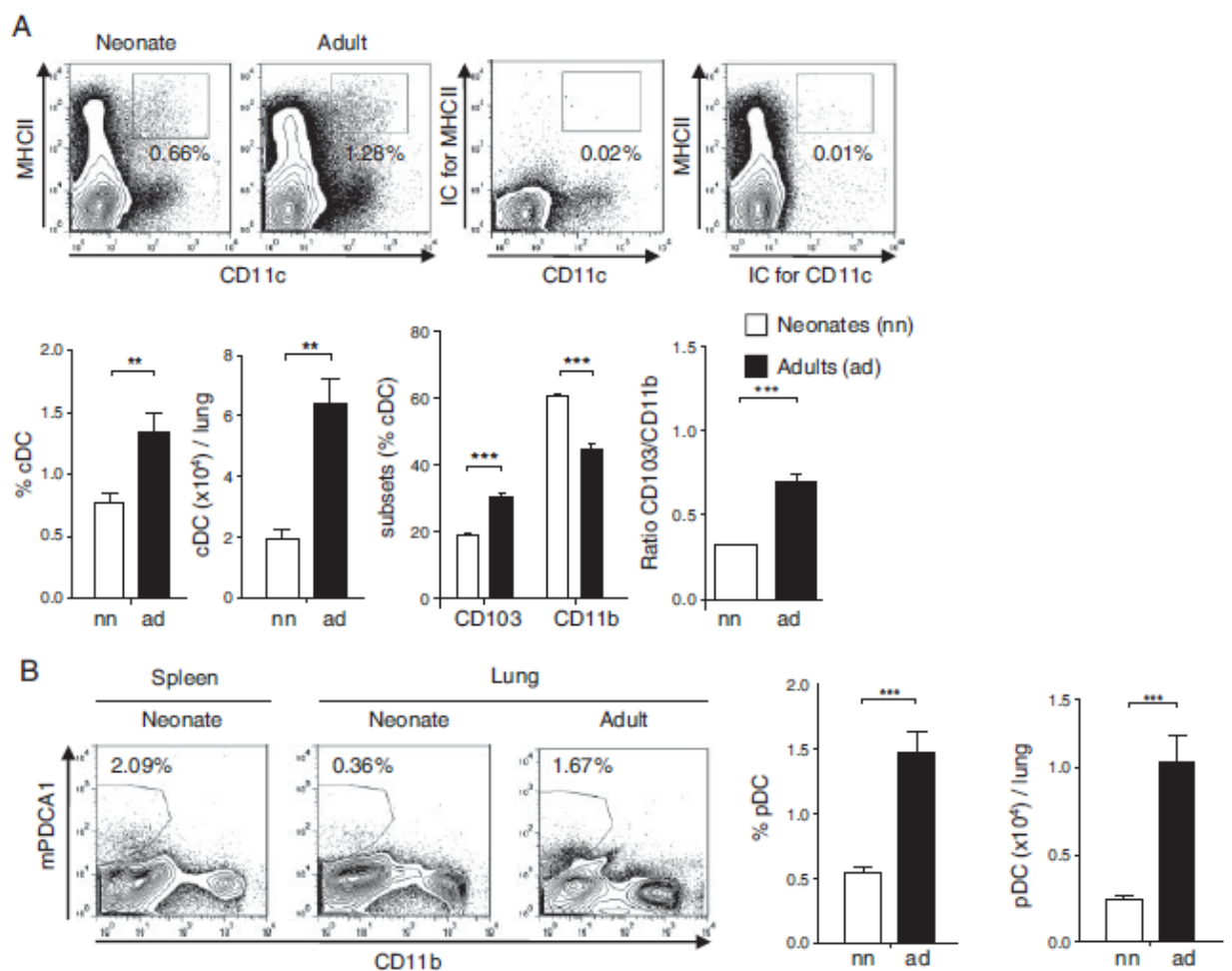
Finally, neonatal and adult lung CD11c<sup>+</sup>MHCII<sup>high</sup> cDCs were sorted 48 h after nasal administration of BCG (BCG-lung-DC) and we investigated their capacity to prime naive CD4<sup>+</sup> T cells. Inguinal lymph nodes (ILN) were chosen as a source of naive CD4<sup>+</sup> T cells since about 87% ILN-CD4<sup>+</sup> T cells were found CD45RB<sup>high</sup> in adults and 94% in neonates (data not shown). Besides, we previously observed that the frequency of naive CD4<sup>+</sup> T cells responding to BCG is high enough in adult and neonatal

BALB/c LNs to monitor their priming upon exposure to BCG-loaded DCs [14, 18]. Control lung cDCs isolated from non-treated adult or neonatal mice did not prime naive CD4<sup>+</sup> T cells for detectable cytokine secretion (data not shown). When neonatal BCG-lung-DCs were co-cultured with naive ILN-CD4<sup>+</sup> T cells isolated from neonatal (1 wk old), weaned (3 wk old) and adult (6 wk old) mice, we observed a progressive increase in the production of IFN- $\gamma$  while the production of IL-5 decreased (Fig. 5D). When adult BCG-lung-DCs were co-cultured with neonatal naive ILN-CD4<sup>+</sup> T cells, the production of IL-5 was predominant over IFN- $\gamma$  whereas they did prime adult naive ILN CD4<sup>+</sup> T cells to produce IFN- $\gamma$  but not IL-5 (Fig. 5D). Thus neonatal CD4<sup>+</sup> T cells seemed to have an inherent Th2 bias.

## Discussion

In the present study we explored in a BALB/c murine model a potential bias of neonatal immune responses at the lung tissue level by monitoring type 1 (IFN- $\gamma$ ) and type 2 (IL-4 and IL-5) cytokine secretion as well as the expression of two transcription factors T-bet and GATA-3, known to be signatures of Th1 and Th2 polarization respectively.

First we showed that, following in vitro CD3 stimulation, neonatal lung explants secreted more Th2 and less Th1 cytokines



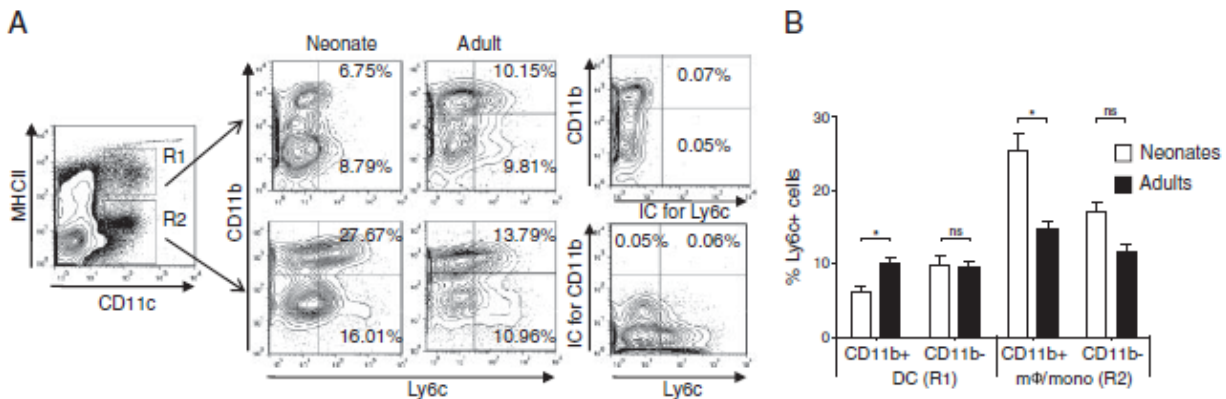
**Figure 3.** Neonate lungs are severely impaired in cDCs and pDCs. Lung cells were isolated from neonatal or adult BALB/c mice and labeled with anti-CD11c, -MHCII (I-A/I-E), -CD11b, -CD103 or -mPDCA1 mAb or matching isotype controls. At least 500 000 events were acquired on a FACS Calibur. Data are representative of four independent experiments. All statistical analysis are carried out using Mann-Whitney U test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). (A) Gating on CD11c<sup>+</sup>MHCII<sup>high</sup> cells allowed comparison of the cDC subset in neonatal versus adult lungs. Representative dot plots and corresponding isotype control staining are shown. The cDCs were analysed for their expression of CD11b and CD103, defining two major subsets CD11b<sup>+</sup>CD103<sup>-</sup> and CD11b<sup>-</sup>CD103<sup>+</sup> (gating strategy and isotype control staining are shown in Supporting Information Fig. 2A). Percentage, total number of cDCs per lung as well as ratio of CD103<sup>+</sup> over CD11b<sup>+</sup> cDCs are shown as histograms. Data are mean  $\pm$  SEM from  $n = 5$ –10 mice. (B) pDCs were identified as mPDCA1<sup>+</sup>CD11b<sup>-</sup> cells (gating strategy and isotype control staining are shown in Supporting Information Fig. 2B). Representative dot plots of spleen or lung pDCs are shown. Percentage and total number of pDCs per lung are depicted for neonate (white bars) and adult (black bars). Data are mean  $\pm$  SEM from  $n = 6$ –12 mice.

and that at basal stage neonatal lungs contained more GATA-3 and less T-bet. Because the polyclonal anti-CD3 activation was performed without co-stimulatory signals such as CD28 or IL-2, the number of responding T cells in lung explants might have been restricted. Finally we showed that neonatal lungs had about three GATA-3<sup>+</sup>CD3<sup>+</sup> cells for each T-bet<sup>+</sup>CD3<sup>+</sup> cell, whereas these numbers were approximately equal in adult lungs. Thus we concluded that the neonatal lung immune environment was characterized by a Th2 bias.

Next, we assessed which CD3<sup>+</sup> lymphocyte subsets were producing GATA-3 and T-bet. Neonatal lungs contained four-fold fewer CD3<sup>+</sup> lymphocytes than adult lungs ( $1.5 \times 10^5$  versus

$7 \times 10^5$  CD3<sup>+</sup> cells respectively). This deficit in T cells appears moderate if compared with that which was previously published for the spleen, as it can be calculated from data in [19] that the neonatal spleen contains 25-fold fewer CD3<sup>+</sup> lymphocytes than the adult spleen ( $25 \times 10^5$  versus  $625 \times 10^5$  CD3<sup>+</sup> cells respectively). Interestingly, we found that neonatal lung CD3<sup>+</sup> cells were significantly enriched in DN CD4<sup>-</sup>CD8<sup>-</sup> cells compared with adult lung. The DN CD3<sup>+</sup> cells could be NKT or  $\gamma\delta$  T cells, both being CD3<sup>+</sup> subsets known to be at least partly CD4<sup>-</sup>CD8<sup>-</sup> in lungs [20, 21]. However, lung NKT or  $\gamma\delta$  T cells were found at lower frequencies than the DN CD3<sup>+</sup> cells, and were not significantly different between adults and neonates.





**Figure 4.** Neonate lungs have an excess of Ly6c<sup>+</sup> monocytic DC precursors. Lung cells isolated from neonatal and adult BALB/c mice were labeled with anti-CD11c, -MHCII (I-A/I-E), -Ly6c and -CD11b mAb or matching isotype controls. (A) Gating on CD11c<sup>+</sup>MHCII<sup>high</sup> cells (R1) or CD11c<sup>+</sup>MHCII<sup>low</sup> (R2) allowed comparison of Ly6c<sup>+</sup> cDCs (upper dot plots) and monocytic DC precursors (lower dot plots) in neonatal versus adult lungs. (Representative dot plots are shown, one experiment with eight mice per group.) (B) Mean percentage and number of the Ly6c<sup>+</sup>, CD11b<sup>-</sup> or CD11b<sup>+</sup> subsets gated within CD11c<sup>+</sup> lung cells (R1 or R2) were determined for neonate (white bars) and adult (black bars). Data are mean ± SEM from n = 4 pools of two mice per group. Statistical analysis was carried out using Mann-Whitney U test (\*p < 0.05).

When staining for GATA-3 among lung CD3<sup>+</sup> lymphocytes, we found an excess of GATA-3<sup>+</sup> cells in neonates associated to the DN CD3<sup>+</sup> subset. Again it appeared unlikely that neonatal GATA-3<sup>+</sup> DN CD3<sup>+</sup> cells were DX5<sup>+</sup> or TCR-γδ<sup>+</sup> since the percentages of GATA-3<sup>+</sup> DX5<sup>+</sup> or TCR-γδ<sup>+</sup> CD3<sup>+</sup> cells were very low (<0.5%) and not significantly different between neonates and adults. Conversely, in adult lung, CD4<sup>+</sup> T cells were, as expected, the main source of GATA-3.

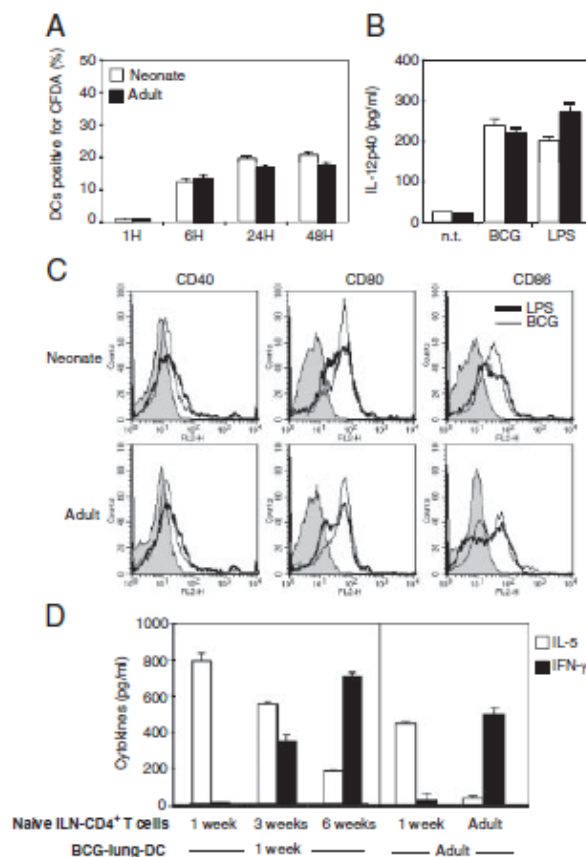
Double-negative T cells comprising 1–5% of all peripheral T cell population of human and mice have been characterized previously as non-conventional inducible regulatory T cells, negative for Foxp3, mainly producing IFN-γ [22, 23]. Whether the GATA-3<sup>+</sup> DN T cells that we have identified in neonate lungs are another subset of DN regulatory T cells remained to be elucidated.

We attempted to look for the transcription factors Foxp3 and RORγt (respectively indicative of Treg and Th17 programming, reviewed in [8]) in neonatal lungs in comparison with adult lungs. Foxp3 (mRNA and protein) was found at lower levels whereas RORγt was found at higher mRNA levels in neonatal lungs (data not shown). Thus we are now seeking to identify RORγt-producing cells in neonatal lungs. Interestingly it has been recently demonstrated that RORγt and GATA-3 co-expressing Th2 memory cells are responsible for asthma exacerbation by producing large amount of IL-17 together with Th2 cytokines [24]. We suggest that the same lung cytokine environment could explain the severity of respiratory diseases in neonates.

DCs are key APCs able to sense and process the signals derived from invasive micro-organisms and to link innate and adaptive immunity. In the present study we addressed the issue of a defect in lung DC subsets in neonatal mice, as was documented for the spleen compartment [25]. Our present study showed a neonatal defect in pulmonary DCs in BALB/c mice for both the cDC and pDC subsets (2 × 10<sup>4</sup> cDCs and 0.25 × 10<sup>4</sup> pDCs in neonates compared with 6.4 × 10<sup>4</sup> cDCs and 10<sup>4</sup> pDCs in adults).

According to previous studies, the neonatal spleen contains 12–50-fold fewer CD11c<sup>+</sup> DCs than adult spleen (15 × 10<sup>4</sup> cDCs in neonates compared with 600 × 10<sup>4</sup> in adults) but pDCs represent 30–40% of all spleen DCs in neonates and only 10–15% in adults [25]. Thus neonatal spleen is enriched in pDCs, which is not the case for neonatal lung (according to our data, pDCs accounted for 11–14% of all lung DCs both in neonates and adults). We also investigated for the first time in neonates the two main cDC subsets, CD11b<sup>+</sup> and CD103<sup>+</sup>, that are present in lungs of adult mice [15]. We clearly showed that BALB/c neonates had a lower CD103<sup>+</sup> to CD11b<sup>+</sup> cDC ratio in their lungs compared with adults. C57BL/6 neonates did also show significant deficiencies both in cDC and pDC subsets in their lungs (Supporting Information Fig. 3A and B). However in the C57BL/6 background the neonatal CD103 to CD11b cDC ratio did not appear different from the adult counterpart (Supporting Information Fig. 3A). Because peripheral CD103<sup>+</sup> cDCs are functionally related to splenic CD8α<sup>+</sup> cDCs [12], our observation that neonatal lungs contained less CD103<sup>+</sup> cDCs, is reminiscent of the delayed accumulation of splenic CD8α<sup>+</sup> cDCs described in 6 day-old pups [5] and could explain why Th2 cells are prominent in the neonatal lungs. Nevertheless splenic CD8α<sup>+</sup> cDCs are resident in lymphoid organs whereas peripheral CD103<sup>+</sup> and CD11b<sup>+</sup> cDCs have to migrate to draining lymph nodes in order to prime naïve T cells. Neonate lung DCs were found to have high levels (even more than adult) of CCR7, the main chemokine receptor for DC migration [16, 26], thus supporting that they are able to migrate to draining LNs.

Lung plasmacytoid DCs are involved in antiviral and anti-inflammatory responses [10, 27]. One can thus speculate that the pDC defect we observed in neonatal mice will compromise their capacity to control pulmonary infection and inflammation. Indeed it has been reported that mice infected as neonates with influenza virus develop persistent pulmonary dysfunction related to deficient priming of CTL [28]. Conversely, treatment of



**Figure 5.** Neonatal and adult lung cDCs display similar functional properties following i.n. administration of LPS or BCG. (A) Phagocytosis of fluorescently labeled live BCG. BCG labeled with CFDA was delivered i.n. to neonatal or adult mice. Phagocytosis was quantified by fluorescent microscopy 1, 6, 24 or 48 h post BCG in the cDC-sorted population ( $CD11c^+MHCII^{high}$ ). (B)  $CD11c^+MHCII^{high}$  DCs were sorted 48 h after i.n. delivery of BCG or LPS to neonatal or adult mice or from non-treated mice (n.t.) and cultured for 24 h in AIM-V medium. IL-12p40 secretion was measured in supernatant (A, B). Data are mean  $\pm$  SEM from  $n = 3$ –5 mice and repeated twice. (C) One day after i.n. administration of BCG or LPS to adult or neonatal mice, lung cells were isolated and stained with anti- $CD11c$ , -I-A/I-E, -CD40, -CD80, and -CD86 mAb or matching isotype controls. 100 000 events were acquired on a FACScan. DCs were gated as  $CD11c^+MHCII^{high}$  as outlined in Fig. 3A. The expression of the co-stimulation markers CD40, CD80 and CD86, after BCG (thin line) or LPS (bold line) stimulation, was compared with isotype control (gray area). (D) BALB/c adult or neonatal mice received BCG i.n. 48 h prior to lung excision. Lung cDCs ( $CD11c^+MHCII^{high}$ ) were then sorted (BCG-lung-DC) and co-cultured 24 h in AIM-V medium with naive ILN- $CD4^+$  T cells of indicated age. IL-5 and IFN- $\gamma$  were quantified by Bio-Plex cytokine assay in the culture supernatants. Data are mean  $\pm$  SEM of triplicates of pooled cells isolated from 10 adults or 16 neonates, and repeated twice.

newborn BALB/c or C57BL/6 mice with Flt3 ligand from day 1 to day 6 after birth increases the number of cDCs (10-fold) and pDCs (3-fold) in spleen and enhances their resistance to a viral challenge with herpes simplex virus up to adult level [29, 30].

In contrast to the deficit in the cDC and pDC populations, we showed that monocytic  $Ly6c^+$  DC precursors were more frequent in neonatal than adult lungs. According to Geissmann et al.,

$Ly6c^+ CD11b^+$  monocytes coming from bone marrow precursors and circulating in the blood can give rise to cDCs in peripheral tissues [13].  $Ly6c^{low}$  monocytes, called “stationary monocytes” by Peng et al. can differentiate into  $CX_3CR1^+$  DCs, which correspond to the pulmonary  $CD11b^+$  cDC subset [31]. Conversely,  $Ly6c^{high} CCR2^{high}$  monocytes can repopulate  $CD103^+$  cDCs in resting lungs [32]. Thus we made the assumption that neonatal lungs have the ability to rapidly expand their cDC population from the resident pool of  $CD11b^+ Ly6c^+$  monocytic precursors.

Finally, we investigated whether neonatal lung cDCs have the capacities of functional APCs at steady state and after in vivo stimulation with strong inflammatory signals such as LPS or BCG. An important feature of functional cDCs is their capacity to produce IL-12 upon stimulation that in turn favors the differentiation of Th1 cells. In our study, neonatal lung DCs, in vivo activated by i.n. administration of BCG or LPS, produced the same amounts of IL-12p40 than their adult counterparts. However, in our conditions neither neonatal nor adult DCs produced IL-12p70 the bioactive form of IL-12 that is usually required for the induction of Th1 immune response. Despite their lack of IL-12p70 production, neonatal and adult lung DCs purified after i.n. delivery of BCG had the same capacity to promote a Th1 immune response when co-cultured with adult naive  $CD4^+$  T cells. It is possible that IL-12p40, produced in large amounts by neonatal and adult lung DCs, forms dimers with a p19 subunit to form IL-23. IL-23 is a member of the IL-12/IL-6 super-family and plays an important role in maintaining Th1 responses [33].

On the basis of these findings, we can postulate that neonatal lung DCs would be able to efficiently prime Th1 immune responses, if adequately stimulated. Indeed, infants vaccinated at birth with BCG produced similar concentrations of IFN- $\gamma$  in response to purified protein derivative and showed similar frequencies of IFN- $\gamma$ -producing lymphocytes as compared with immune adults, thus demonstrating that the human neonatal immune response to BCG is not biased toward Th2 [34].

Conversely, we showed that both neonatal and adult lung DCs matured in vivo upon i.n. exposure to BCG, primed in vitro neonatal naive  $CD4^+$  T cells toward Th2 cytokine secretion pattern. This finding supports the view that neonatal  $CD4^+$  T cells, at least the naive one in lymphoid organs, are intrinsically programmed along the type 2 profile. An illustration of a deleterious neonatal Th2 imprinting can be drawn from an experimental mouse model of airway disease eosinophilic exacerbation caused by the RSV in both BALB/c and C57BL/6 genetic backgrounds [35, 36]. Treatment of newborn BALB/c mice with an anti-sense IL-4R $\alpha$  oligonucleotide was sufficient to restore a Th1 response to primary neonatal infection with RSV and suppress the eosinophilic disease exacerbation upon reinfection at adult age [37].

Acute lower respiratory infections represent one of the main health problems among children under five years of age. We hope that our present data will help bringing new insights into the mechanisms leading to Th2-mediated airway disease upon neonatal respiratory infection and the conditions required to stimulate Th1 immunity following vaccination in neonates.



## Materials and methods

### Animals

BALB/c mice were bred and housed under EOPS conditions in our animal facilities (INRA Jouy-en-Josas or Pasteur Institute, Paris) or purchased from the Centre d'Élevage Janvier (Le Genest, St. Isle, France). At the time of experimentations, neonates were 5–6 days old and adults were 6–8 wk old. Animal experiments were carried out under the authority of license issued by the Direction des Services Vétérinaires (Dr. Riffault, accreditation number 78-27) and followed national guidelines for animal welfare.

### Immunizations

BCG strain 1173P2 used to immunize neonatal and adult mice was produced in Pasteur Institute as previously described [38] and administered i.n. to neonatal mice ( $10^5$  CFU in 5  $\mu$ L) and to adult mice ( $10^6$  CFU in 30  $\mu$ L). In some experiments, neonatal and adult mice received 15  $\mu$ g (5  $\mu$ L) and 60  $\mu$ g (30  $\mu$ L) of LPS (Sigma) respectively. The doses of BCG and LPS were optimized in preliminary experiments. Control neonates and adults received PBS (5 and 30  $\mu$ L, respectively).

### Cytokine production by lung explants

Lungs of neonatal and adult mice were perfused via the right ventricular cavity, excised and cut into small pieces of 3–5 mm maximal dimensions. These lung explants were cultured in AIM-V medium (Invitrogen) with or without 1  $\mu$ g/mL soluble anti-CD3. Supernatants were harvested after 6 and 24 h of culture for IL-5 and IFN- $\gamma$  assays respectively, as these time points were shown in a previous study to generate maximal signal with limited background in the Bio-Plex Cytokine assay [14]. Explants were dried, weighed and results are expressed as pg/mL of cytokines secreted per 10 mg of dry lung tissue.

### Transcription factors in cell extracts

Total proteins (45  $\mu$ g) extracted from neonatal or adult whole lung cells were resolved on 7.5% SDS-PAGE and transferred to nitrocellulose sheets. Blots were probed with mAb anti-T-bet, anti-GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti- $\beta$ -actin (Ac-15 Abcam, Cambridge, UK) followed by HRP-conjugated polyclonal goat anti-rabbit (Dako Cytomation, Denmark) or goat anti-rabbit IgG (Santa Cruz Biotechnology). The immune complexes were revealed by enhanced chemiluminescence detection system (Amersham, France). For data acquisition and background normalization, we used ScanAlyze software version 2.50 as previously described [39]. Data were expressed as the ratio (target proteins versus  $\beta$ -actin) of the normalized intensities of bands.

### Lung cell isolation

After broncho-alveolar lavages were performed by gentle intratracheal instillation of PBS, EDTA 1 mM (0.5 or 1 mL respectively, for neonates and adults), neonatal and adult lungs were perfused via the right ventricular cavity of the heart with, respectively, 5 and 10 mL of 0.9% NaCl and aseptically removed. Single lung cell suspensions were prepared by enzymatic digestion of the tissue as previously described [14] and resuspended in AIM-V medium supplemented with 10% FCS.

In some experiments, when lungs were collected 24 or 48 h after i.n. administration of BCG or LPS, lung cells were further enriched in DCs using CD11c microbeads and an AutoMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany).

### FACS analysis

For FACS analysis, lung cells were stabilized in Cyto-Chex (Strek, Omaha, NE), and stored at 4°C up to 7 days. Lung cells were first incubated with anti-CD32/CD16 (FcBlock, BD bioscience) prior to staining with FITC, PE, PerCP or biotin-conjugated MAb reactive to CD3, CD4, CD8, DX5, TCR- $\gamma\delta$ , CD11c, CD11b, CD103, CD80, CD86, CD40, MHCII (IA/IE), CCR7 (BD Biosciences), to mPDCA1 or Ly6c (Miltenyi Biotec). Streptavidin conjugated to allophycocyanin (BD Biosciences) was used as secondary staining for biotinylated mAb. Intracellular staining with anti-T-bet and -GATA-3 mAb conjugated to PE (eBiosciences) was performed using BD Cytotfix/Cytoperm™ solution according to manufacturer's instructions. Matched isotype control mAbs were used throughout. Data were acquired with an FACS Calibur (BD Biosciences) and analyzed with the FlowJo Software v7.5 (Tree Star Inc, San Carlos, CA). At least 500 000 events (up to 2 millions) were acquired for each sample.

### Labeling of live BCG and cell purification after phagocytosis

To determine the phagocytic capacity of lung DC, carboxyfluorescein-diacetate succinimidyl ester (CFDA; Molecular Probes, Eugene, OR) was used to label live BCG bacteria as previously described [14]. At various time points after i.n. delivery of CFDA-BCG, lung CD11c<sup>+</sup>/MHCII<sup>high</sup> cDCs were purified using an FACSstar or an FACSaria cell sorter (BD Biosciences). Sorted DCs (purity >95%) were cytocentrifuged ( $10^5$  cells/slide) and stained with Diff-Quick (Baxter Dade AG, Duedingen, Switzerland). The number of cells that were significantly phagocytic (2–3 fluorescent bacteria per cell) was expressed as a percentage of total cells counted (200–400 cells counted per slide).

### IL-12p40 production by lung cells

Forty-eight hours after i.n. delivery of BCG, CD11c<sup>+</sup>/MHCII<sup>high</sup> DCs were sorted as described in the previous section and cultured

without any stimulation in AIM-V medium supplemented with 10% FCS at 37°C and 5% CO<sub>2</sub>. Supernatants were harvested after 24 h and IL-12p40 was measured by the Bio-Plex Cytokine assay (Bio-Rad, Marnes La Coquette, France) according to manufacturer's instructions.

### Source and enrichment of naïve CD4<sup>+</sup> T lymphocytes and co-cultures with lung DCs

Inguinal LNs of 1-, 3- and 6-wk-old naïve mice were excised and single-cell suspensions were prepared. CD4<sup>+</sup> T were sorted with MACS CD4<sup>+</sup> microbeads and an AutoMACS separator, according to manufacturer's instructions and co-cultured at a ratio 5:1 with neonatal or adult lung CD11c<sup>+</sup>/MHCII<sup>high</sup> DCs, FACS-sorted 48 h after i.n. delivery of BCG. The mixed DC and T cell cultures were incubated in triplicate in flat-bottomed 96-well plates (Nunc) for 72 h at 37°C, 5% CO<sub>2</sub> then IFN-γ and IL-5 were assayed in the supernatant by the Bio-Plex Cytokine test.

### Statistical analysis

Statistical analyses were performed with GraphPadPrism v5 software, using the non-parametric Mann–Whitney *U* test (two-tailed) to compare two unpaired groups or Student's *t*-test in experiments with triplicate samples, as mentioned in the figure legends. Values of *p* < 0.05 were considered statistically significant (ns: non-significant; \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001 and \*\*\*\**p* < 0.0001). Data are given as mean ± SEM.

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**Abbreviations:** cDC: conventional DC · DN: double negative · ILN: inguinal lymph node · MHCII: MHC class II · pDC: plasmacytoid DC · RSV: respiratory syncytial virus

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# Nucleoprotein Nanostructures Combined with Adjuvants Adapted to the Neonatal Immune Context: A Candidate Mucosal RSV Vaccine

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## Abstract

**Background:** The human respiratory syncytial virus (hRSV) is the leading cause of severe bronchiolitis in infants worldwide. The most severe RSV diseases occur between 2 and 6 months-of-age, so pediatric vaccination will have to be started within the first weeks after birth, when the immune system is prone to Th2 responses that may turn deleterious upon exposure to the virus. So far, the high risk to prime for immunopathological responses in infants has hampered the development of vaccine. In the present study we investigated the safety and efficacy of ring-nanostructures formed by the recombinant nucleoprotein N of hRSV (N<sup>RSV</sup>) as a mucosal vaccine candidate against RSV in BALB/c neonates, which are highly sensitive to immunopathological Th2 imprinting.

**Methodology and Principal Findings:** A single intranasal administration of N<sup>RSV</sup> with detoxified *E. coli* enterotoxin LT(R192G) to 5–7 day old neonates provided a significant reduction of the viral load after an RSV challenge at five weeks of age. However, neonatal vaccination also generated an enhanced lung infiltration by neutrophils and eosinophils following the RSV challenge. Analysis of antibody subclasses and cytokines produced after an RSV challenge or a boost administration of the vaccine suggested that neonatal vaccination induced a Th2 biased local immune memory. This Th2 bias and the eosinophilic reaction could be prevented by adding CpG to the vaccine formulation, which, however did not prevent pulmonary inflammation and neutrophil infiltration upon viral challenge.

**Conclusions/Significance:** In conclusion, protective vaccination against RSV can be achieved in neonates but requires an appropriate combination of adjuvants to prevent harmful Th2 imprinting.

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## Introduction

Human respiratory syncytial virus (hRSV) is a major cause of severe lower respiratory tract infections in infants less than 6 months and in immuno-compromised or elderly patients [1,2,3,4]. Besides, infants who develop acute RSV bronchiolitis at an early age are at increased risk for prolonged wheezing and future development of asthma [5,6] through excessive priming of Th2 cells [7]. Bovine RSV (bRSV) is a closely related pneumovirus, also causing severe and sometimes fatal respiratory disease in calves [8].

Veterinary RSV vaccines exist but could be improved, whereas several factors have impeded the development of an effective and safe hRSV vaccine [9,10]. First of all, concerning a pediatric RSV vaccine, safety will be of peculiar concern since, in the 60's, a vaccination trial with formaldehyde-inactivated virus in alum (FI-RSV) led to an exacerbated disease upon seasonal RSV infection

in most vaccine recipients and two infants died with a prominent pulmonary neutrophilia and moderate eosinophilia [11,12]. Extensive studies using rodent models have attributed disease exacerbation to immunopathological responses: in adult BALB/c mice, Th2 biased immune responses to the FI-RSV vaccine lead, upon RSV challenge, to a pro-inflammatory "cytokine/chemokine storm" promoting excessive pulmonary leukocyte infiltration, with prominent eosinophilia, and goblet cell hyperplasia [13]. Second, apart from the elderly, one major target population for vaccination is the newborn or very young infant, with its relatively immature innate and adaptive immune system prone to Th2-biased immune responses [14], and potential interference of maternal antibodies [15]. Third, adjuvants and/or delivery modes best adapted to elicit protective and non pathogenic mucosal immunity in such very young infants have yet to be improved and licensed. Interestingly, studies in neonatal mice have suggested ways to reduce the Th2



bias of neonatal responses to sub-unit protein vaccines, like the use of CpG oligodeoxynucleotides as adjuvant [16,17].

Most of the RSV vaccines presently under development are targeting the two surface glycoproteins F and G, which bear epitopes recognized by neutralizing antibodies [10]. Much less has been done to explore the potential interest as a vaccine component of the nucleoprotein (N), although it was recognized as one of the targets of T cell immunity, inducing both helper and cytotoxic T cells in human [18,19]. Besides, N is highly conserved between hRSV subtypes and even bears >85% amino-acid homology with N from bRSV, so it could be an interesting component of a heterosubtypic vaccine. Indeed, the combination of plasmids encoding the RSV N and F proteins administered to calves or infant rhesus monkeys was shown to provide protection without causing disease exacerbation [20,21].

An original process was developed in our laboratory, allowing to produce and purify large amounts of recombinant N from hRSV as soluble ring structures composed of 10–11 N monomers bound to random stretches of bacterial RNA (70 bp), which we named N<sup>SRS</sup> (for Sub-nucleocapsid Ring Structure) [22,23]. In a recent paper, we documented its immunogenicity and vaccine potential, when administered to adult BALB/c mice with, as adjuvant, the mutant *E. coli* heat-labile toxin LT(R192G) (hereafter abbreviated as LT) [24]. Nasal vaccination with N<sup>SRS</sup> and LT elicited strong local and systemic immunity characterized by high titers of anti-N antibodies (IgA in the broncho-alveolar lavage (BAL) and serum IgG1 and IgG2a), antigen specific CD8+ memory T cells and IFN $\gamma$  producing CD4+ T cells in the absence of pathological lung inflammation upon RSV challenge [24]. The same antigen administered parenterally with a water-in-oil adjuvant (Seppic) to colostrum-deprived one-month-old calves proved partially protective against bRSV challenge, without inducing lung eosinophilia or adverse lung inflammation [25].

In the present study, we chose to test our new mucosal vaccine candidate in newborn (5 to 7 days old) BALB/c mice, a very relevant animal model to address the risk of immuno-pathological responses against RSV [26]. Indeed, a primary RSV infection in BALB/c neonates leads to airway disease exacerbation with lung eosinophilia upon re-infection at adult age [27]. Besides, FI-RSV vaccination of BALB/c neonates has a more severe immunopathological outcome upon virus challenge than the one elicited upon FI-RSV vaccination of adult BALB/c mice [28]. We confirmed the capacity of our vaccine to trigger efficient anti-viral immunity after just one nasal administration to 5–7 days old pups. However viral protection was associated with some disease exacerbation and airway infiltration with neutrophils and eosinophils upon RSV challenge, that appeared related to a Th2 neonatal imprinting. Addition of a second pro-Th1 adjuvant (CpG oligodeoxynucleotides) was able to restore a mixed Th1/Th2 immune memory and abrogated eosinophilia with only a minor reduction of viral protection upon challenge. Surprisingly, neonates treated with this combination of adjuvants without antigen turned out to be equally protected against virus replication.

## Results

### Neonatal nasal vaccination with N<sup>SRS</sup> and LT(R192G) provided viral protection but exacerbated lung disease upon challenge

Five to seven days old pups received one intranasal (i.n.) administration of N<sup>SRS</sup> (3  $\mu$ g) with or without adjuvant LT (2  $\mu$ g) (this vaccine formulation is noted thereafter N+LT). Mice vaccinated as neonates were challenged 4 to 5 weeks later with hRSV-A2. Mice were killed at day 5 post-challenge, which

allowed us to monitor the viral load, the weight loss and the onset of the antibody response or lung inflammation in the same individual mice. Pups for one experimental group were chosen from at least two different litters and to reach a sufficient number of pups in each experimental group allowing statistical analysis, we combined data from several experiments (each experiment is shown with a different symbol).

A single neonatal N+LT i.n. immunisation led to a strong reduction of the viral mRNA load in the lungs 5 days after challenge (Fig. 1A, N+LT *versus* unvaccinated RSV-infected controls C+  $p < 0.001$ ). Administered alone, LT did not reduce the viral load and N gave partial and highly variable protection (Fig. 1A, N *versus* C+  $p < 0.05$ ).

As illustrated Fig. 1B, the RSV challenge induced a moderate but significant weight loss from day 2 up to day 5 post-infection in 5 weeks-old mice (all infected groups *versus* uninfected littermates [C-]  $p < 0.001$ ). Weight loss was more severe in the N+LT vaccinated group than in unvaccinated (C+) or N vaccinated mice (N+LT *versus* C+  $p < 0.01$ ; N+LT *versus* N  $p < 0.001$ ). Neonatal vaccination with LT alone also led to increased weight loss in most mice (LT *versus* N  $p < 0.001$ , but LT *versus* C+ or N+LT  $p > 0.05$ ) suggesting a contribution of LT to disease exacerbation.

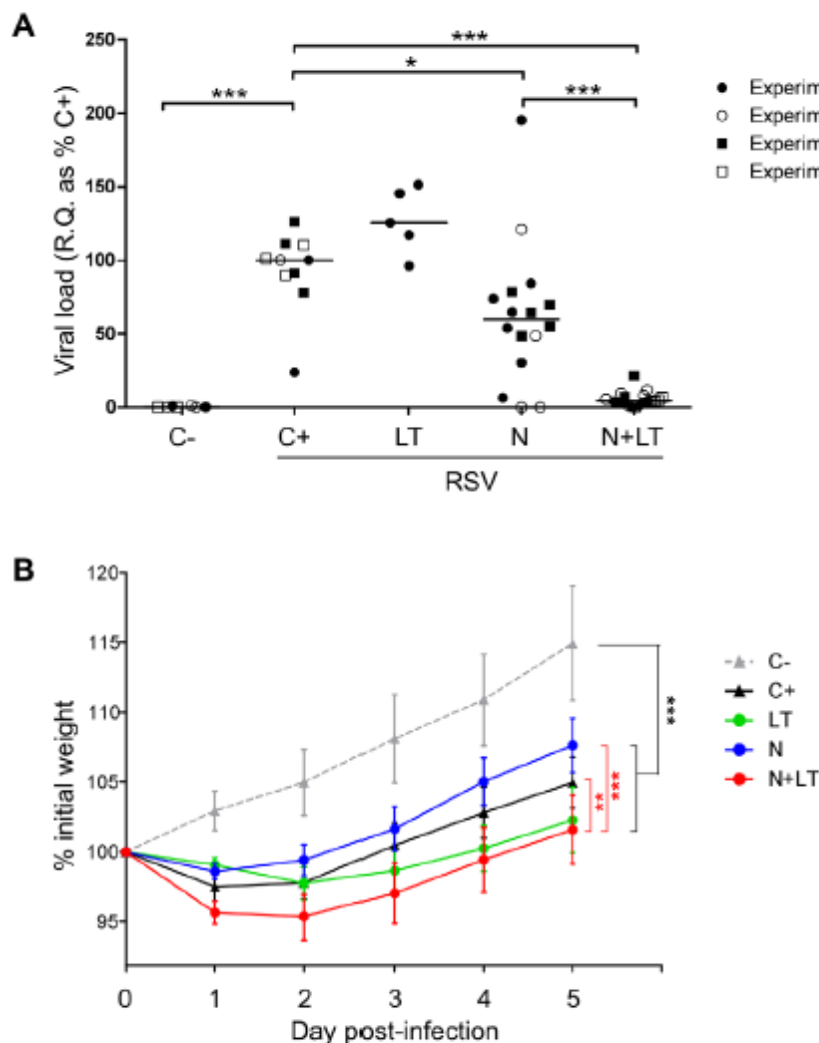
Moreover, mice vaccinated as neonates with N+LT and challenged with RSV as young adults had significantly more leukocytes in their BAL at day 5 p.i. than unvaccinated infected controls (C+) or mice vaccinated with N alone (Fig. 2A: N+LT *versus* C+  $p < 0.001$ ). Vaccination with LT alone also led to an increased number of BAL leukocytes in most mice upon challenge, although to a more variable and thus non significant level (LT *versus* C+  $p > 0.05$ ).

Determination of BAL cell subtypes by May-Grünwald-Giemsa staining showed that the viral challenge *per se* did not alter significantly BAL cell composition, except for a minor but significant increase in the number of lymphocytes (Fig. 2B: C+ *versus* C-  $p < 0.0001$ ). Vaccination with N or LT alone led to a further and highly significant increase in BAL lymphocytes (LT *versus* C+  $p < 0.001$ ; N *versus* C+  $p < 0.01$ ), neutrophils (Fig. 2C: LT *versus* C+  $p < 0.01$ ; N *versus* C+  $p < 0.001$ ) and eosinophils (Fig. 2D: LT *versus* C+  $p < 0.0001$ , N *versus* C+  $p < 0.001$ ). Combining N+LT in the neonatal vaccine further increased the number of lymphocytes, neutrophils and eosinophils (N+LT *versus* C+  $p < 0.0001$  for the 3 subsets). Taken together, these data suggested that both LT and N contributed to the inflammatory responses recorded after the RSV challenge. As pulmonary eosinophilia was suggestive of a Th2 biased memory response to N, we explored the type of antiviral immunity primed by N+LT and investigated further whether it could be modulated.

### Neonatal vaccination with N+LT imprinted a Th2 biased N-specific immunity, which could be modulated with CpG

Sera collected before (d0) and 5 or 8 days after the viral challenge (d5, d8 p.i.) were titrated by ELISA for anti-N antibodies. As illustrated Fig. 3, anti-N antibody titers were at baseline level in all groups before challenge (a month after neonatal vaccination) but increased as early as 5 days and even more so at 8 days after the RSV challenge in all N+LT vaccinated mice, suggesting a memory response.

To further explore this hypothesis, we amplified the primary neonatal response by a vaccine boost with N at 5 weeks. As shown in Table 1 for N+LT primed mice, serum antibodies detected 7 days after the boost were mostly of the IgG1 subclass and no anti-N IgA were detected in the BALF. To reduce the Th2 neonatal imprinting, we decided to implement our neonatal vaccine



**Figure 1. Neonatal nasal vaccination with N+LT conferred viral protection but exacerbated airway disease upon RSV challenge.** Male and female pups (5–7 day-old) were vaccinated by intranasal instillation of 10  $\mu$ L saline containing or not 3  $\mu$ g N and 2  $\mu$ g LT as indicated. At 5 weeks of age, mice were challenged by intranasal instillation of 50  $\mu$ L ( $5.10^6$  pfu) hRSV A2. Controls included unvaccinated infected (C+) and uninfected (C-) littermates. Animals were killed 5 days post challenge (d5 p.i.). (A) Individual viral load assessed by qRT-PCR: R.Q. of N transcripts, normalized to HPRT, are expressed as % of the unvaccinated infected control group (C+) ( $R.Q. = 100 \times 2^{-\Delta\Delta Ct}$ ). Four independent experiments combining different treatment groups are shown with  $\geq 5$  mice per group (C-: n=7, C+: n=10, LT: n=5, N: n=16, N+LT: n=20). Mann-Whitney U-test was used for comparison between treatments (\*  $p < 0.05$ ; \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ). (B) Mice were weighed daily from d0 till d5 p.i. and individual weight loss/gain was calculated as % of initial weight. Data are mean  $\pm$  SEM from  $n \geq 5$  mice per group. Statistical analysis was performed to compare growth over the period d2 to 5 p.i. using the Tukey's multiple comparison test, repeated measures one way ANOVA (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ).

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formulation with CpG (ODN-1826), a known pro-Th1 adjuvant in neonates [16]. Indeed, contrarily to their N+LT primed littermates, a few mice primed with N+CpG and most mice primed with N+LT+CpG had high titers of IgG2a anti-N serum antibodies, as well as detectable anti-N IgA in their BALF 7 days after the N boost (Table 1).

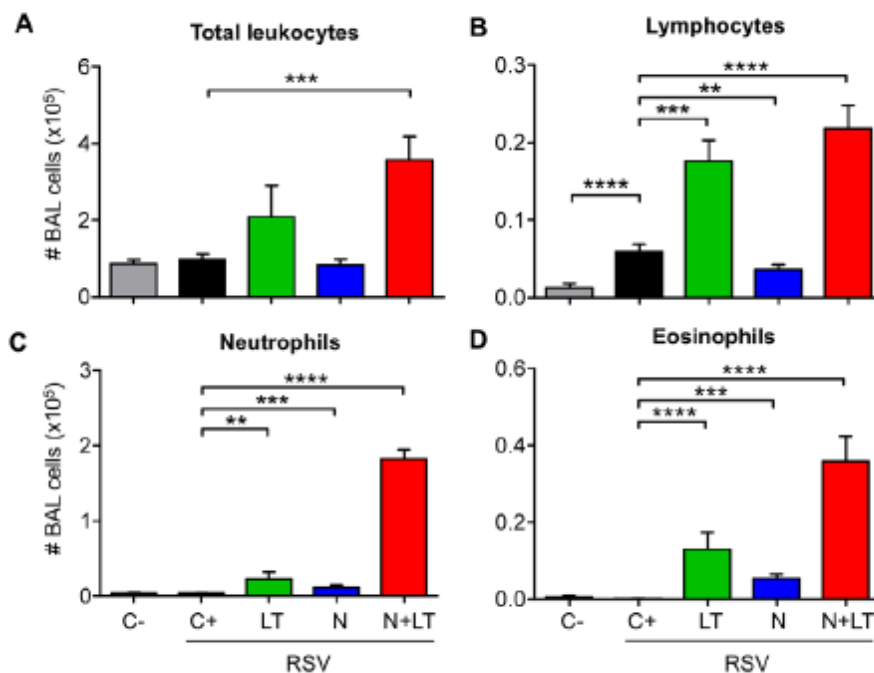
Spleen and local lymph node (LN) cells, collected 7 days after a boost with N, were cultured for 72 h *in vitro* with N, or with PMA-Ionomycin and medium alone as positive and negative controls respectively. Culture supernatants were assayed by ELISA for IFN $\gamma$ , a Th1 cytokine and IL-5, a Th2 cytokine. As illustrated Table 2, spleen and LN cells from N+LT primed pups boosted with N produced both IFN $\gamma$  and IL-5, with the highest IL-5 titers being produced by LN cells in some mice, confirming that

neonatal N+LT vaccination induced a Th2 biased local T cell memory. By contrast, spleen and LN cells from mice primed with N+LT+CpG or N+CpG produced higher titers of IFN $\gamma$  than of IL-5, confirming that CpG added to the neonatal N+LT vaccine reoriented memory T cells towards Th1 (Table 2).

#### Combining CpG and LT as adjuvants for neonatal vaccination provided protection against viral challenge without lung eosinophilia

In order to test whether CpG added to the neonatal N or N+LT vaccine could also prevent disease exacerbation after challenge, neonates vaccinated i.n. with N and/or LT and/or CpG were challenged with RSV a month later.

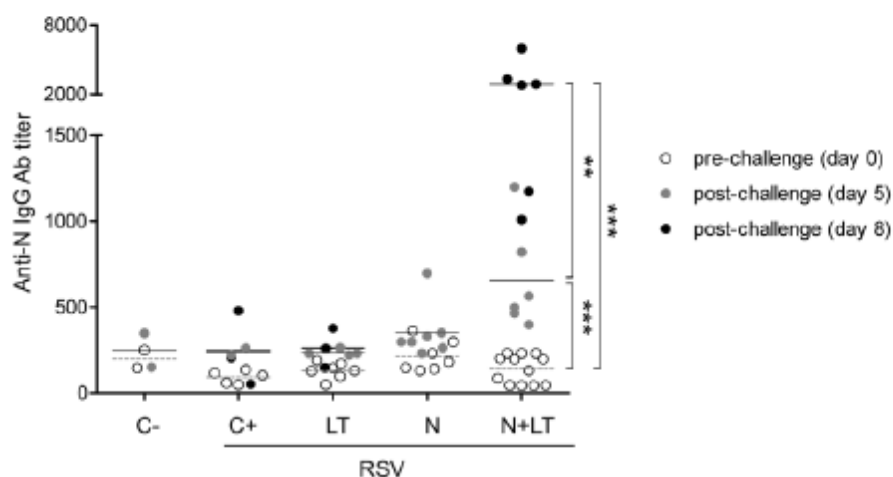




**Figure 2. LT and N contributed to the inflammatory responses recorded in BAL after the RSV challenge.** Mice vaccinated as neonates as in Fig.1 were killed 5 days post challenge (d5 p.i.). BAL cells were enumerated, cytocentrifuged and stained with May-Gründwald-Giemsa (MGG). Data are mean  $\pm$  SEM from  $n \geq 10$  mice per group for total leukocytes (A), lymphocytes (B), neutrophils (C) and eosinophils (D) numbers. Mann-Whitney U-test was used for comparison between treatments (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ). doi:10.1371/journal.pone.0037722.g002

Addition of CpG to each vaccine formulation tested (LT, N and N+LT) was associated with a moderate increase in the number of BAL leukocytes, that was not significant except between the groups vaccinated with N+CpG versus N ( $p < 0.05$ , Fig. 4A). Addition of CpG led to a slight increase in the number of lymphocytes (except when added to LT alone, Fig. 4B), and, whereas it did not reduce the number of neutrophils (Fig. 4C), it nearly abolished BAL eosinophilia after challenge in each

formulation (Fig. 4D, N+CpG versus N  $p < 0.01$ , LT+CpG versus LT and N+LT+CpG versus N+LT  $p < 0.0001$ ). FACS analysis of BAL cell suspensions (Fig. 4E) confirmed that addition of CpG to the N+LT neonatal vaccine abolished BAL eosinophilia (from nearly 8% down to 0.3% SiglecF<sup>+</sup> CD11c<sup>low</sup> CD45<sup>+</sup> BAL cells). As expected, BAL leukocytes were mostly alveolar macrophages (SiglecF<sup>+</sup>, CD11c<sup>+</sup>) for CpG vaccinated controls, whereas a variable proportion of BAL leukocytes were double negative



**Figure 3. Neonatal N+LT nasal immunization primed for an early anti-N Ab response after the RSV challenge.** Mice vaccinated as neonates as in Fig.1 were bled before (day 0) and 5 or 8 days after the viral challenge with hRSV-A2 (d5 or d8 p.i.). Serum anti-N antibody titers were assessed by an endpoint dilution ELISA assay on plates coated with N using HRPO-conjugated rabbit anti-mouse Ig(H+L) Abs. Individual titers and the mean titer for day 0 (white circles, dotted line), d5 p.i. (grey circles, plain line) and d8 p.i. (black circles, plain line) are figured. Man-Whitney U-test was used for comparison of titers at day 0, 5 and 8 p.i. for each group (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). doi:10.1371/journal.pone.0037722.g003

**Table 1.** Anti-N antibody responses primed by neonatal nasal vaccination shifted from IgG1 to IgG2a and IgA isotypes when CpG were added as adjuvant.

Prime <sup>(a)</sup>	Serum		BALF			
	IgG1	IgG2a	IgG1	IgG2a	IgA	
N+LT	1694±836 <sup>(b)</sup>	4/4 <sup>(c)</sup>	53±19	1/4	≤3	0/5
N+CpG	493±395	3/6	3751±3397	1/6	5±2	1/6
N+LT+CpG	764±396	9/9	1523±1252	5/9	10±1	6/9
LT+CpG	46±12	2/8	≤30	0/8	≤3	0/2

<sup>(a)</sup>Neonates were immunised *i.n.* at age 5–7 days as indicated (prime) and received one intranasal boost with N (10 µg) at 5 weeks. Sera and BALF were collected 7 days after the boost.

<sup>(b)</sup>Antibody titers against N were determined by ELISA using endpoint dilution assay. Data are mean±SEM (≤30 or ≤3: not detected at the first tested dilution for sera or BALF).

<sup>(c)</sup>Number of responders/number tested.

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(lymphocytes or neutrophils) for the N+CpG, N+LT or N+LT+CpG vaccinated groups.

Histological examination of lungs collected 8 days post-challenge (Fig. 5) showed few diffuse peribronchiolar infiltrates in the lungs from C+ (data not shown) or LT+CpG vaccinated mice (Fig. 5A, 5D). On the opposite, lung sections from mice vaccinated with N+LT (Fig. 5B, 5E) or N+LT+CpG (Fig. 5C, 5F) displayed large peribronchiolar and perivascular immune infiltrates mostly composed of mononuclear cells, which clearly suggested the development of bronchus associated lymphoid tissue (iBALT). Although the average iBALT area/lung section ( $n \geq 2$  mice/group) was not statistically different (Fig. 5G), eosinophils (indicated with red asterisk) were frequently observed at the periphery of these infiltrates in lung sections from N+LT (Fig. 5E) but were rarely found in lung sections from N+LT+CpG vaccinated pups (Fig. 5F).

We then investigated whether vaccine formulation could modify the pattern of expression of several chemokines that could affect the Th1/Th2 balance and the recruitment of eosinophils. The levels of expression of mRNAs encoding the murine chemokines mCCL2 (MCP1), mCCL11 (Eotaxin-1), mCCL3 (MIP1 $\alpha$ ) and mCCL5 (RANTES) were assessed by quantitative real time PCR in lungs collected 5 days after the viral challenge (Fig. 6). As opposed to mice administered CpG alone in the neonatal period, which tended to have reduced levels of all four chemokine transcripts after challenge, N+LT+CpG vaccinated mice had significantly increased levels of CCL2 and CCL3 mRNA (Fig. 6A and 6C), whereas N+LT vaccinated mice had significantly increased levels of CCL11 (Fig. 6B) compared to the other groups (N+LT *versus* C+  $p < 0.05$ ; N+LT *versus* N+LT+CpG  $p < 0.01$ ). This observation is consistent with the recruitment of BAL eosinophils following challenge in N+LT vaccinated mice as opposed to their absence when CpG were added to the vaccine formulation. The level of expression of CCL5 was not significantly affected by vaccination (Fig. 6D).

We next tested whether addition of CpG to the vaccine formulation influenced weight loss, antibody production or viral load after an RSV challenge. Adding CpG to the neonatal N+LT vaccine abrogated the increase in weight loss after the RSV challenge (N+LT *versus* N+LT+CpG  $p < 0.05$ ) (Fig. 7A). N+LT primed mice produced mostly IgG1 anti-N antibodies as early as 5 days after the viral challenge (Fig. 7B). Addition of CpG to N or N+LT primed for an early rise in IgG2a anti-N antibodies,

**Table 2.** Memory T cell responses primed by neonatal nasal vaccination shifted from Th2 to Th1 cytokine profile when CpG were added as adjuvant.

Prime <sup>(a)</sup>	Spleen <sup>(b)</sup>		LN <sup>(b)</sup>	
	IFN $\gamma$ <sup>(c)</sup>	IL-5	IFN $\gamma$	IL-5
N+LT	654±159 <sup>(d)</sup>	9±6	1431±650	511±361
N+CpG	614±20	<7±0	667±97	112±78
N+LT+CpG	2268±734	7±2	580±112	64±46
LT+CpG	357±98	7±3	182±123	8±5

<sup>(a)</sup>Neonates were immunised *i.n.* at age 5–7 days as indicated (prime) and received one intranasal boost with N (10 µg) at 5 weeks.

<sup>(b)</sup>Spleen and cervical lymph node (LN) were collected 7 days after the boost (LN were pooled from 2 to 3 mice). Single cell suspensions were cultured with N, PMA-ionomycin or medium for 72 hours.

<sup>(c)</sup>Culture supernatants were assayed by ELISA for IFN $\gamma$  and IL5.

<sup>(d)</sup>Cytokine concentrations in supernatants from cultures restimulated with N (mean pg/mL ±SEM for  $n \geq 2$  for spleen and pool of LN). (All cell cultures responded to PMA, producing 39838±8542 pg/mL IFN $\gamma$ ).

doi:10.1371/journal.pone.0037722.t002

resulting in a more balanced IgG2a/IgG1 response in some mice, although the differences between groups were not statistically significant, due to highly variable individual titers.

Finally, as illustrated on figure 7C, neonatal administration of CpG alone significantly decreased the viral load in the lungs on day 5 p.i. (CpG *versus* C+  $p < 0.05$ ) and combining CpG with LT even led to an almost complete “non-specific” protection against challenge (LT+CpG *versus* C+  $p < 0.01$ ). Under these conditions, adding N to CpG or LT+CpG did not significantly improve protection although the level of protection elicited by N+LT vaccination remained slightly better (N+LT *versus* N+LT+CpG  $p < 0.05$ ) (Fig. 7C).

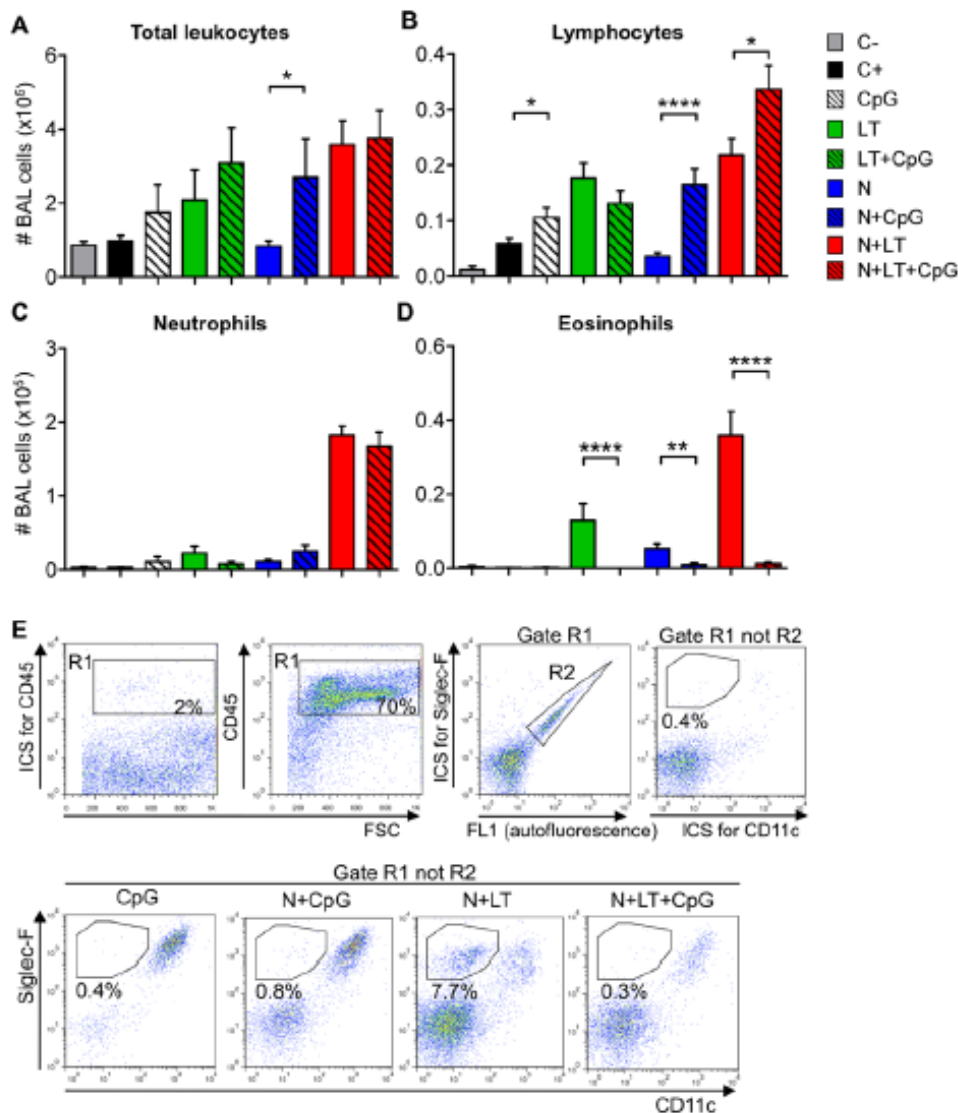
Thus efficient protection against RSV during the neonatal period could be achieved by combining the non-specific stimulatory effects of LT and CpG and antigenic priming with N.

## Discussion

In the present study we investigated in BALB/c neonate mice the safety and efficacy of a new mucosal vaccine candidate against RSV, which we previously validated in adult BALB/c mice [24]. BALB/c neonatal mice ( $\leq 7$  days of age) have been considered as best approximating immune maturity of  $\leq 2$  months old babies [29] an age when RSV vaccination should be undertaken, since the most severe RSV disease affects mostly 2 to 6 month-old infants. Several studies addressing the issues of optimizing conditions for neonatal vaccination and, in particular, reducing the Th2 bias of neonatal immune responses have been performed using BALB/c neonates [16,30]. Moreover BALB/c neonates constitutes a most sensitive experimental model for assessing immunopathological imprinting as generated by FI-RSV vaccination [28] or by an early RSV infection [26]: the propensity of the host to develop a skewed Th2 response to RSV at an early age set the stage for subsequent development of an asthma-like phenotype on re-exposure to the same virus [27,31,32].

Our present data show that neonatal mucosal vaccination with N+LT can generate efficient viral protection, resulting in over 95% reduction of the viral load, 5 days after a viral challenge with RSV at early adult age. Yet, this neonatal vaccination induced a prolonged state of hyper-reactivity to the virus, as shown by an increased weight-loss and an infiltration of the broncho-alveolar compartment by neutrophils and eosinophils a few days after the



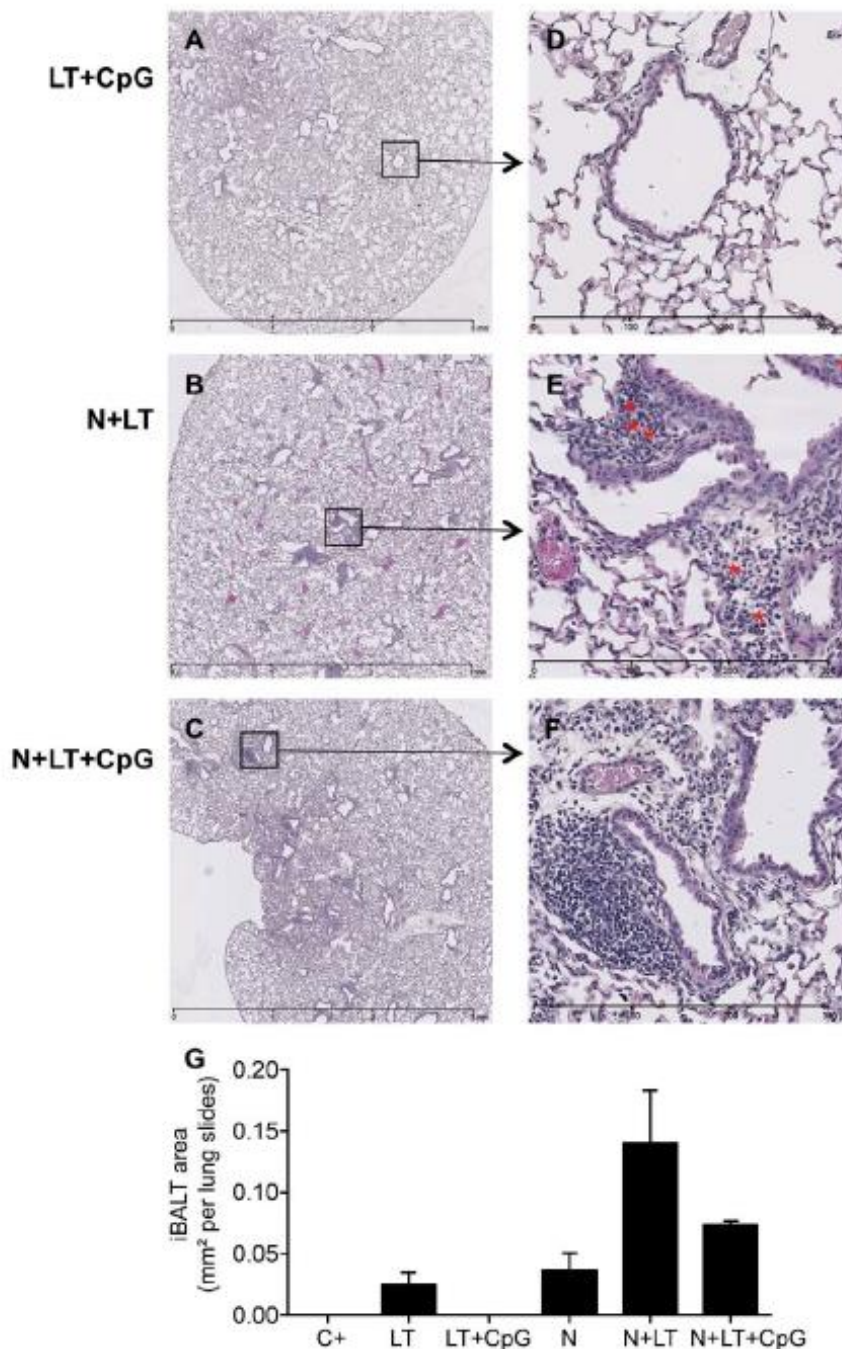


**Figure 4. CpG added to the neonatal vaccine abrogated BAL eosinophilia upon RSV challenge.** Mice vaccinated as neonates as in Fig. 1, with or without addition of 2 nmoles CpG (ODN 1826), as indicated, were sacrificed 5 days after the viral challenge with hRSV-A2. BAL leukocytes were enumerated, cytocentrifuged and stained with May-Grünwald-Giemsa (MGG). Data are mean  $\pm$  SEM from  $n \geq 10$  mice per group combining three independent experiments. Mann-Whitney test was performed to compare the total number of leukocytes (A), as well as the number of lymphocytes (B), neutrophils (C) and eosinophils (D) (\*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ). (E) BAL cells from each experimental group were pooled ( $n \geq 5$  mice per group) and stained for FACS analysis using Siglec-F-PE, CD45-PerCP and CD11c-Biotin monoclonal antibodies followed by Streptavidine APC. Isotype control stainings (ICS) were done with irrelevant isotype-matched antibodies on a pool of BAL cells from all experimental groups. After gating on CD45<sup>+</sup> leukocytes (R1) and excluding autofluorescent cells (R2), eosinophils were detected as Siglec-F<sup>+</sup> and CD11c<sup>low</sup>. Data analysis was performed using FlowJo software with at least 5,000 events in the (R1 not R2) gate. Dot plots represent one of two experiments with similar data. doi:10.1371/journal.pone.0037722.g004

viral challenge. No such signs of vaccine-enhanced disease were observed in our first evaluation of the N+LT vaccine in adult BALB/c mice [24], which underscores the usefulness of the neonatal BALB/c mouse model for assessing the safety of a new RSV vaccine candidate, as previously stated by Plotnicky et al [28].

Individual weight-losses post-challenge were moderate (rarely over-passing 5–10% for 2–3 days), as compared to 15–20% weight-loss after challenge if mice had been primary infected as 4–6 days-old pups ([27] and our own unpublished data) or vaccinated as adults with M2 peptide with another mutant of LT (LT-K63) as adjuvant [33]. Yet, average weight-loss was significantly higher (over the period d2 to d5 p.i.) in the N+LT

vaccinated group as compared to N-vaccinated or unvaccinated mice. As this suggested some vaccine (or adjuvant) exacerbated disease, we monitored lung inflammation by differential enumeration of the BAL leukocytes: clearly both the LT(R192G) adjuvant and, concerning eosinophils, the N<sup>SRS</sup> antigenic component of the neonatal vaccine contributed to the recruitment of granulocytes in the broncho-alveolar space after the viral challenge, which did not occur in unvaccinated age-matched infected controls. Again, the magnitude of this inflammatory reaction was far below that seen in neonatally infected mice reinfected at adult age (e.g. eosinophilia reaching over 35% of BAL cells, [27,31] and our own unpublished data). Comparing a large set of data from individual mice belonging to our different vaccination groups, we found a highly



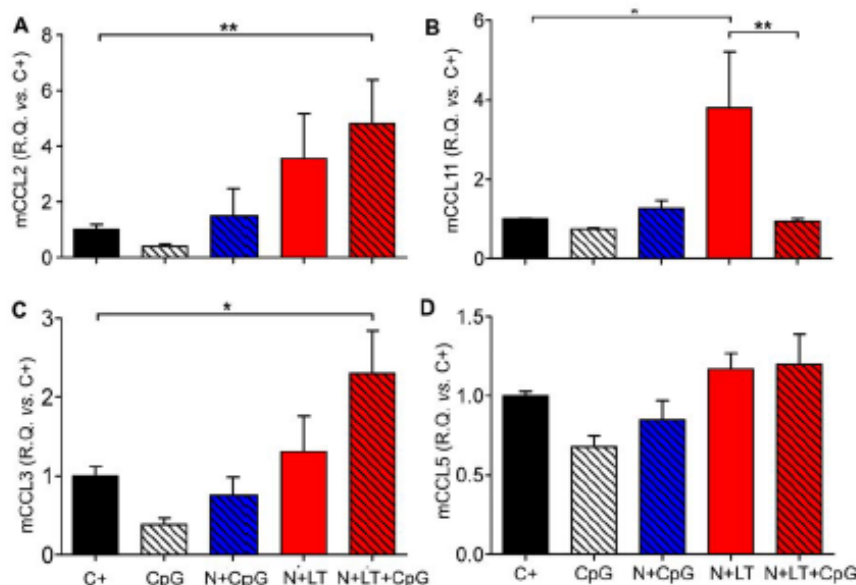
**Figure 5. Neonatal nasal vaccination with N+LT or N+LT+CpG augmented cellular infiltration in lung tissue upon RSV challenge.** Mice vaccinated as neonates as indicated or non vaccinated littermates were sacrificed 8 days after the hRSV-A2 challenge. Lung were dissected out, fixed, embedded in paraffin and sectioned at 5  $\mu$ m. Lung sections were then stained with hematoxylin-eosin-saffron and photographed using a Nanoscope (Hamamatsu). One representative section per group is shown (A–C: original magnification 2.5 $\times$ ) with red asterix figuring eosinophils in the enlarged selected area (D–F: enlargement 20 $\times$ ). (G) Total iBALT area was measured using the NDPview software (Hamamatsu) for one representative lung section of each mouse ( $n \geq 2$  mice per group). Data are mean  $\pm$  SEM. doi:10.1371/journal.pone.0037722.g005

significant inverse correlation between individual viral load and % PMN cells in BAL ( $p < 0.0001$  for neutrophils,  $p < 0.01$  for eosinophils). This suggested that vaccination has set the stage for inflammatory responses that could contribute to protection upon viral challenge. Interestingly eosinophils have been proposed to promote RSV clearance [34] and were exonerated from

contributing to vaccine-enhanced disease in experiments undertaken in adult mice [35,36].

Anti-N antibodies were rarely detected in the serum of N or N+LT vaccinated mice one month after neonatal vaccination, but an early anti-N antibody response could clearly be induced in most N+LT vaccinated mice by an RSV challenge or by a boost with N.





**Figure 6. CpG added to the neonatal vaccine decreased pulmonary CCL11 mRNA expression upon RSV challenge.** The level of expression of genes encoding mCCL2 (A), mCCL3 (C), mCCL5 (D) and mCCL11 (B) was monitored by quantitative real time PCR of RNAs extracted from individual lungs collected at d5 p.i. Data were normalized to the mHPRT and expressed relative to the unvaccinated infected control group (C+) (R.Q. =  $100 \times 2^{-\Delta\Delta Ct}$ ). Data are mean  $\pm$  SEM from  $n \geq 4$  mice per group (Mann Whitney test, \*  $p < 0.05$ ). Two independent experiments were done. doi:10.1371/journal.pone.0037722.g006

Thus, provided the LT adjuvant was co-administered,  $N^{SRS}$  was immunogenic in neonates and this N-specific antibody response could be boosted by an RSV infection. The almost exclusive production of IgG1 antibodies suggested that the immune memory resulting from neonatal N+LT vaccination was Th2 biased. Yet, we did not detect anti-N IgE antibodies upon neonatal vaccination, nor after the vaccine boost or viral challenge (data not shown), as was the case after a primary neonatal RSV infection followed by a challenge at adult age ([37] and our own unpublished data).

That neonatal i.n. vaccination with N+LT also primed for N-specific memory T cells was confirmed by the fact that spleen and respiratory lymph node cells isolated a week after a vaccine boost secreted both  $IFN\gamma$  and IL-5 upon *in vitro* restimulation with N. Once again the relatively high amount of the Th2 cytokine IL-5 secreted by local lymph node cells, suggested that the neonatal priming with N+LT had opened the way to a Th2 biased local memory.

Our experimental settings are very similar to those reported by VanCott *et al.* who vaccinated i.n. 7 d old BALB/c pups with the recombinant VP6 protein of EDIM rotavirus together with the same LT(R192G) adjuvant and induced a substantial level of protection against an oral EDIM virus challenge administered one month after vaccination [38]. Neither serum IgG/IgA nor stool IgA anti-VP6 antibodies could be detected in most mice before challenge, as was the case for anti-N antibodies in our experiments. However, splenocytes collected one month after the neonatal vaccination, before any vaccine boost or viral challenge, were shown to contain and/or secrete mostly  $IFN\gamma$ , and almost no IL-5, leading the authors to suggest that protection involved immune Th1 cells.

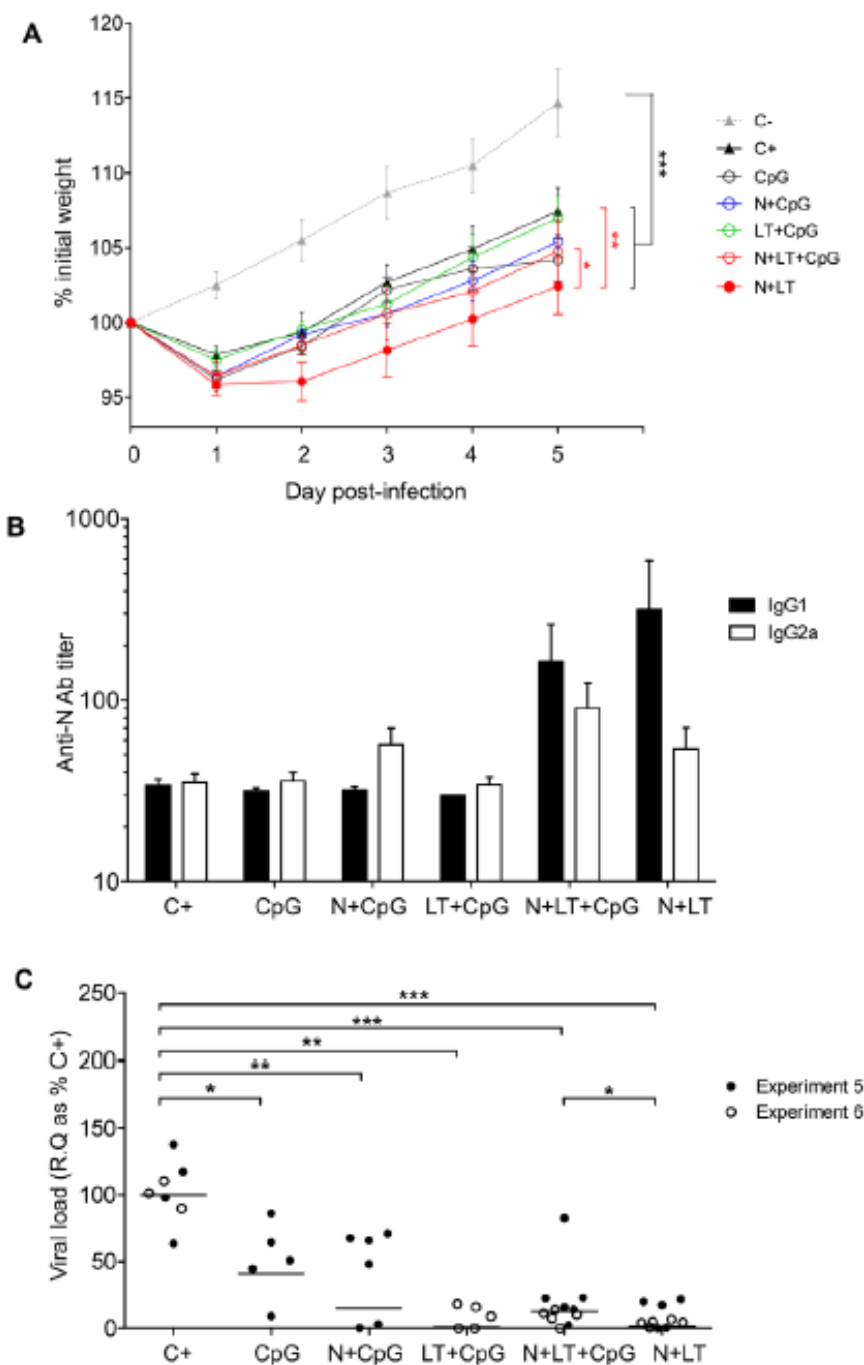
In many other studies, however, vaccination of BALB/c neonates with peptide or protein antigens led to Th2 biased immune responses [39]. Furthermore, primo-infection with hRSV in BALB/c neonates has also been shown to induce Th2 biased immune responses: mediastinal lymph node cells from mice primo-

infected as pups respond with high IL-5 and reduced  $IFN\gamma$  secretion upon restimulation in culture with RSV infected, irradiated syngeneic splenocytes, in opposition to the responses of spleen or LN cells from mice primo-infected as adult [40].

Several mechanisms have been proposed at the molecular level to explain the Th2 bias of BALB/c neonates. First, the Th2 cytokine locus is hypomethylated during the neonatal period, thus allowing rapid high-level Th2 gene transcription (*IL-4*, *IL-5* and *IL-13*) [41]. Second, neonatal memory Th1 cells express high level of interleukin 13 receptor  $\alpha 1$  (*IL-13R $\alpha$ 1*) which heterodimerizes with *IL-4R $\alpha$* , making Th1 cells prone to apoptosis driven by IL-4 produced by activated Th2 cells [42]. Finally, we have recently demonstrated that lung neonatal T cells have Th2 features and that the number of conventional and plasmacytoid dendritic cells is severely reduced in neonatal lungs [43].

Nevertheless it is possible to achieve Th1 immune responses in the neonatal context. For instance, several studies showed that addition of CpG to a neonatal vaccine can help to reduce the Th2 bias of the immune response [16,17,44]. Indeed adding CpG to our N+LT neonatal vaccine not only reduced the Th2 bias of the recall response to a boost with N (less IL-5, more IgG2a), but also abrogated eosinophilia following an RSV challenge, as assessed in BAL cells. These observations are highly consistent with the significant decrease in mCCL11 (eotaxin) mRNA that we have recorded in the lungs when CpG were added to the N+LT vaccine.

However addition of CpG to the N+LT vaccine did neither reduce the neutrophilia in BAL post-challenge, nor the inflammatory leukocytic infiltrates in lung sections. Correlations have been reported between the pattern of chemokine production and the severity of RSV disease in infants [45,46] or with the Th1/Th2 immunopathological imprinting induced by RSV infection after prior sensitization to individual RSV proteins (F or G) in adult BALB/c mice [47]. According to these studies, the enhanced level of expression of CCL2 and CCL3 mRNA upon challenge of our N+LT and N+LT+CpG vaccinated mice, is consistent with mixed



**Figure 7. CpG added to the neonatal vaccine reduced weight loss, increased secondary IgG2a response and maintained viral protection upon RSV challenge.** Mice vaccinated as neonates as indicated or non vaccinated littermates (C+) were sacrificed 5 days after the hRSV-A2 challenge. Data from 2 independent experiments combining different treatment groups are shown with  $\geq 5$  mice per group (C+:  $n=7$ , CpG:  $n=5$ , N+CpG:  $n=6$ , LT+CpG:  $n=5$ , N+LT:  $n=10$ , N+LT+CpG:  $n=11$ ). (A) Mice were weighed daily from d0 till d5 p.i. and individual weight loss/gain was calculated as % of initial weight. Statistical analysis was performed to compare growth over the period d2 to d5 p.i. using the Tukey's multiple comparison test, repeated measures one way ANOVA (\*  $p<0.05$ ; \*\*  $p<0.01$  and \*\*\*  $p<0.001$ ). (B) IgG1 and IgG2a anti-N antibodies were titrated by ELISA in sera collected 5 days after the challenge. Data are mean  $\pm$  SEM from  $n \geq 5$  mice per group. (C) Individual viral load in lungs: R.Q. of N transcripts, normalized to HPRT, are expressed as % of the mean of unvaccinated infected control group (C+) (R.Q. =  $100 \times 2^{-\Delta\Delta C_t}$ ). Data from 2 independent experiments combining different treatment groups are shown. Mann-Whitney U-test was used for comparison between treatments (\*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$  and \*\*\*\*  $p<0.0001$ ). doi:10.1371/journal.pone.0037722.g007

Th2 (increase in CCL2 mRNA) and Th1 (increase in CCL3 mRNA) inflammatory settings, and could explain the increased number of BAL neutrophils. Interestingly, CCL3 was recently

shown to protect against TNF $\alpha$ -mediated weight-loss and illness after a primary RSV infection in adult mice [48].

Both N+LT and N+LT+CpG neonatal vaccination conferred highly significant reduction of the viral load. However, the level of



protection achieved in the presence of CpG could be partly related to some innate effect on the neonatal immune system since CpG and even more so LT+CpG alone very significantly reduced lung viral load, extending a previously described observation by Cho and al. [49]. Nevertheless, N+CpG vaccinated mice were not protected against RSV replication as efficiently as were the N+LT or N+LT+CpG vaccinated mice, suggesting that a combination of the stimulation of innate immunity by CpG, favoring Th1 responses, and the stimulation of N-specific immunity by the LT adjuvant, both contributed to protection.

$N^{SRS}$  are nanostructures which could by itself confer them innate immunostimulatory properties: indeed, protein nanocages of quite similar structure and size delivered intranasally to adult mice were shown to induce iBALT development and to confer protection against several respiratory viruses [50]. Accordingly, in our own experiments, histological examination of lungs collected early after the RSV challenge showed clear development of iBALT along the main bronchi, if mice had been vaccinated as pups with N+LT or with N+LT+CpG. Interestingly, neonatal mice were recently shown to be particularly prone to iBALT induction upon inflammatory (LPS or CpG) stimuli, with IL-17 being essential to iBALT development in the neonatal period [51].

Thus, although intranasal vaccination may seem a logical route for protection against respiratory pathogens because it will efficiently prime local immune effectors, it can also potentiate inflammatory responses through local priming of Th17 memory cells [52]. We did not look for these immune mediators in the present study but we are keen to evaluate other delivery route like the intradermal or transcutaneous routes, able to generate systemic and local immunity with increased safety and feasibility in the neonatal context [53]. Significantly, a double LT mutant (dmLT) with even lower toxicity than LT(R192G) has been developed [54]. Finally, we currently explore whether our  $N^{SRS}$  nanoparticles could be used as carrier to improve immunogenicity of peptides encoding major RSV neutralizing epitopes from F and G envelope proteins.

Thus we believe that a safe and efficient pediatric vaccine against RSV will require innovative solutions through adapted combination of antigen, adjuvant and delivery route. Our present work confirms that safety and efficacy of RSV vaccine candidates should be evaluated in the most sensitive neonatal models.

## Materials and Methods

### Ethics statement

All animal experiments were carried out under the authority of licence issued by the Direction des Services Vétérinaires (accreditation number 78-115 to A. P.-C.) and approved by the ethics committee COMETHEA (COMité d'ETHique appliqué à l'Expérimentation Animale INRA Jouy-en-Josas et AgroParis-Tech): authorization number 11/036. All efforts were made to minimize suffering.

### Vaccine formulation and virus

The production and purification of the recombinant N protein from hRSV (Long strain) in *E. coli*, as sub-nucleocapsid ring structures of 10-11 monomers of N bound to random stretches (70 bases) of bacterial RNA ( $N^{SRS}$  referred as N) have been described previously [23,24]. For use as a vaccine, an additional filtration on EndoTrap red® columns (Hyglos GmbH, Germany) reduced endotoxin contamination to <1 EU/mg protein (QCCL-1000 LAL assay, Cambrex).

The detoxified mutant *E. coli* enterotoxin LT(R192G), provided endotoxin free by Prof. Clements (Tulane University, New

Orleans, USA) was kept sterile at 4°C. Phosphorothioate- CpG oligodeoxynucleotides (ODN) 1826 (TCCATGACGTTTCCT-GACGTT) was purchased from Sigma Genosys, resuspended in injectable water as a 1 mM stock solution and kept frozen at -20°C.

The hRSV strain A2 (provided by Prof. Openshaw, Imperial College, Saint Mary's Hospital, London) was propagated and titrated by plaque assay on HEp-2 monolayers using avicel-containing overlay media to control virus spread. Five days after infection, lived cells were stained with crystal violet and plaque forming units (pfu) were numerated.

### Animals and immunization-challenge protocols

Male and female BALB/c mice purchased from the Centre d'Élevage Janvier (Le Genest, St Isle, France) were bred and housed under FELASA pathogen-free conditions in our animal facilities (IERP, INRA, Jouy-en-Josas).

Five to seven days old pups of both sexes were vaccinated by intranasal instillation of 8 to 10  $\mu$ L of 0.9% endotoxin-free NaCl, containing or not 3  $\mu$ g  $N^{SRS}$  and/or 2  $\mu$ g LT(R192G) and/or 2 nmoles (12.7  $\mu$ g) CpG ODN 1826 (Sigma-Aldrich). Each treatment was administered to 4 to 8 pups from at least 2 different litters, in order to minimize individual variations.

Since a limited number of pups could be included, handled and infected at the same time in any given experiment, all desired control groups could not be included in each experiment. Hence, experimental data presented in the figures usually comprise data from two or more independent experiments.

At 5 to 6 weeks of age, mice were anesthetized by an intraperitoneal injection of Avertine (2.5 mg/g of body-weight of 2-2-2-tribromoethanol, Aldrich) and received, by intranasal instillation either a vaccine boost (10  $\mu$ g  $N^{SRS}$  in 25  $\mu$ L of saline), or a challenge RSV infection (50  $\mu$ L,  $5.10^6$  p.f.u. of hRSV-A2). Individual daily weight was recorded until mice were killed 5 or 8 days after challenge, which allowed us to monitor the viral load, the weight loss and the onset of the antibody response and lung inflammation in the same individual mice.

### Sample collection

Sera were obtained from blood collected via retro-orbital puncture before (d0) and 7 days after a boost immunization, or 5 to 8 days after a challenge infection.

Mice were killed by cervical dislocation and the spleen and local lymph nodes (LN) (cervical and maxillary LN draining the upper respiratory tract and mediastinal LN) were dissected out in complete RPMI medium (RPMI 1640, Gibco BRL, supplemented with 10% heat-inactivated FCS, 2  $\mu$ M L-Glutamine, Penicillin and Streptomycin).

The left bronchus was clamped and the left lobe of the lungs was snap-frozen in liquid nitrogen, and kept frozen at -80°C until processed for RNA extraction for qRT-PCR assays.

Broncho-alveolar lavages (BAL) were performed by flushing the other lobes of the lungs via tracheal puncture four times in and out with 0.7 mL  $Ca^{2+}$  and  $Mg^{2+}$ -free PBS supplemented with 1 mM EDTA. BAL fluids were centrifuged and supernatants (BALF) were stored frozen at -20°C for ELISA assays of anti-N antibodies or cytokines. BAL cells were resuspended in complete RPMI medium and adjusted to appropriate concentrations for cyto-centrifugation or FACS analysis.

Alternatively, the lungs were gently filled with 0.5 mL of 10% paraformaldehyde (PFA) via the trachea and preserved in 10% PFA at 4°C until processed for histological examination.

### Determination of pulmonary RSV viral load and chemokine expression by quantitative Real-Time PCR

Frozen lungs (left lobes) were resuspended in 0.6 mL lysis buffer (Qiagen RTL buffer plus 0.1% 2-mercaptoethanol, Merck) in V bottomed microtubes containing 0.1 mL of 1 to 1.2 mm ceramic beads (Mineralex, Bron, France) (both RNase free) and vortexed for 15 s at 6000 rpm using a bead grinder homogenizer (Precellys 24, Bertin Technologies, Ozyme, St Quentin en Yvelines, France). Total RNA were extracted from lung homogenate using RNA minikit columns (Qiagen), with a 15 min DNase treatment (RNase free DNase set, Qiagen), according to the manufacturer's instructions. Eluted RNA were quantified at 260 nm using a Nanodrop (Labtech, France). Individual RNA samples (4 µg) were reverse transcribed for 1 h at 42°C, using 300 U of M-MLV Reverse Transcriptase (SuperScript II, Invitrogen) with 7 µM of random hexanucleotide primers (pd(N)<sub>6</sub>, Pharmacia Biotech), 15 nmol of each dNTP, 5 mM DTT and 60 U of ribonuclease inhibitor (RNaseOUT, Invitrogen) according to the manufacturer's instructions.

The primers (from Sigma-Aldrich, aliquoted and stored frozen as 100 µM solutions at -20°C) used to target either a conserved region of the *N* gene (position 42 to 125 of huRSV A2, [24]), or the murine *HPRT* gene, or the *CCL2*, *CCL3*, *CCL5* and *CCL11* murine chemokine genes (design with <http://mouseprimerdepot.nci.nih.gov/>) are listed in Table 3.

Real time PCR was run in 96 well microplates on a Perkin Elmer ABI Prism 7900HT Sequence Detector. For determination of the viral load or of *CCLx* gene expression, individual cDNA (1 µL diluted in 10 µL RNase-free water) were mixed with 15 µL SYBR GREEN PCR Master Mix (Applied Biosystems) containing 300 nM of both *N* primers, 500 nM of both *CCLx* primers or 500 nM of both *HPRT* primers, in triplicate for each gene. Non-template controls were run in each assay. Fluorescence curves were analyzed using the software Sequence Detector System (SDS 2.3, Perkin-Elmer) to determine the cycle threshold (Ct) values for each gene. Individual data were normalized to *HPRT*, by calculating the  $\Delta Ct$  value  $\{Ct(N \text{ or } CCLx) - \text{median } Ct(mHPRT)\}$  and the  $\Delta\Delta Ct$   $\{\text{sample } \Delta Ct - \text{mean } \Delta Ct \text{ of at least 3 unvaccinated infected controls (C+)}\}$  were calculated for each sample. Viral load was expressed as % of unvaccinated infected controls (using the formula  $R.Q. = 100 \times 2^{-\Delta\Delta Ct}$ ). The viral load data from our qRT-PCR assay showed strong correlation with those from our tissue culture plaque assay (Fig. S1,  $r = 0.99$ ,  $p < 0.001$ ).

Similarly, the expression of the chemokine transcripts normalized to *mHPRT* transcripts are expressed relative to unvaccinated infected controls (C+) ( $R.Q. = 2^{-\Delta\Delta Ct}$ ).

### May-Grünwald-Giemsa staining and histology

BAL cells from individual mice, were enumerated, spread on duplicate microscope slides (Superfrost, Thermo, France) by cytocentrifugation (5 min, 700 rpm, Cytospin 5, Shandon, France) and stained with May-Grünwald and Giemsa. At least 400 leukocytes were counted blindly for each sample.

Lungs collected d8 p.i. were fixed in 10% paraformaldehyde, embedded in paraffin and 5 µm sections, stained by hematoxylin, eosin and saffron were photographed using a Nanozoomer (Hamamatsu).

### N-specific antibody E.L.I.S.A

Individual mouse sera and BALF were assayed for N-specific antibodies (Ig(H+L), IgG2a, IgG1 or IgA) by ELISA as previously described in [24]. End-point antibody titers were calculated by regression analysis, plotting dilution versus A450 using Origin software (regression curve  $y = (b+cx)/(1+ax)$ ). Endpoint titers were defined as the highest dilution resulting in an absorbance value twice that of non-immune control sera or BALF.

### IFN-γ and IL-5 production by immune spleen or lymph node cells

Red-blood cell depleted leukocyte suspensions were prepared as previously described [24] from individual spleens or lymph nodes (LN: cervical and mediastinal pooled from 2 to 3 mice) collected 7 days after a boost with N. Spleen or LN cells resuspended in RPMI complete medium and 50 µM 2-Mercaptoethanol were seeded in 96 wells microplates (4.10<sup>5</sup> cells/well) and co-cultured in triplicates with N (10 µg/mL) or PMA-Ionomycin (10 ng/mL and 1 µg/mL, respectively) or culture medium. After 72 h at 37°C with 5% CO<sub>2</sub>, supernatants for each condition were pooled and stored frozen at -20°C.

Supernatants were assayed for IFNγ and IL-5 by standardized ELISA assays as previously described [24]. Briefly, for IFNγ we used mAb R4-6A2 for capture and biotinylated XMG1.2 for revelation (BD biosciences) and murine rIFNγ (R&D systems) diluted from 3300 to 1.5 pg/mL in duplicate wells to establish a standard curve. For IL-5, we used mAb TRFK5 for capture and biotinylated TRFK4 (BD biosciences) for revelation and murine rIL5 (R&D systems) diluted from 1000 to 7.5 pg/mL in duplicate wells to establish a standard curve. Cytokine concentrations were determined using the Revelation software (Dynex).

### Flow cytometry

After a first 20 min blocking step using anti-CD32/CD16 (FcBlock, BD bioscience, 5 µg/ml or 10 µg/ml for highly inflammatory BAL collected post-infection), BAL cells were labeled with anti-CD45-PerCP, anti-Siglec-F-PE and anti-

**Table 3.** List of primers used for quantitative real time PCR.

Name of gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
N (huRSV-A2)	AGATCAACTTCTGTATCCAGCAA	TTCTGCACATGATAATTAGGAGTATCAAT
mHPRT	CAGGCAGACTTTGTTGGAT	TTGGCTCATCTTAGGCTTT
mCCL2	GGGATCATCTTGCTGGTGAA	AGGTCCTGTGATGCTCTG
mCCL3	GATGAATTGGCGTGGAACTCT	CTGCCCTTGCTGTCTCTCTC
mCCL5	CCCACCTCTCTCTGGGTTG	GTGCCACGTCAAGGAGTAT
mCCL11	TAAAGCAGCAGGAAGTTGGG	CATCTGTCTCCCTCCACCAT

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CD11c-biotin, followed by streptavidin-APC (all from BD Pharmingen).

Cells were then fixed in 10% Cellfix (BD bioscience) and data, acquired on at least 5,000 CD45+ leukocytes with a FACSCalibur (BD Biosciences), were analyzed with Flow Jo software. After gating on CD45+ leukocytes and excluding autofluorescent cells, BAL eosinophils were gated as Siglec-F+, CD11c<sup>low/-</sup> according to [55].

### Statistical data analysis

Data were expressed as arithmetic mean  $\pm$  standard error of the mean (SEM). Non-parametric Mann-Whitney (two groups), ANOVA Tukeys multiple comparison test (>2 groups) or Spearman correlation statistical tests were used to compare unpaired values (GraphPadPrism software). Values of  $p < 0.05$  were considered significant, levels of significance are indicated on the graphs with stars: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; and \*\*\*\*  $p < 0.0001$ .

### Supporting Information

**Figure S1 Correlation between viral load titration by plaque assay and q-RT-PCR.** Five infected and two non-infected adult mice were sacrificed 4 days after the hRSV-A2 challenge. The lungs were cut in two equal parts and the viral load

was titrated either by plaque assay on HEp-2 monolayers, or by qRT-PCR: R.Q. of N transcripts, normalized to HPRT, are expressed as % of the infected mice (C+) ( $R.Q. = 100 \times 2^{-\Delta\Delta Ct}$ ). Non parametric Spearman correlation test was used to calculate a correlation coefficient ( $r$ ), (\*\*\*  $p < 0.001$ ). (TIIF)

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### Author Contributions

Conceived and designed the experiments: AR XR JFE SR APC. Performed the experiments: AR XR CD JF SB MM APC. Analyzed the data: AR XR SB SR APC. Contributed reagents/materials/analysis tools: JF SB MM JFE. Wrote the paper: AR XR SR APC.

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# Flt3 ligand improves the innate response to respiratory syncytial virus and limits lung disease upon RSV reexposure in neonate mice

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Respiratory syncytial virus (RSV) causes severe bronchiolitis in infants worldwide. The immunological factors responsible for RSV susceptibility in infants are poorly understood. Here, we used the BALB/c mouse model of neonatal RSV infection to study the mechanisms leading to severe disease upon reexposure to the virus when adults. Two major deficiencies in neonatal lung innate responses were found: a poor DCs mobilization, and a weak engagement of the IFN<sub>1</sub> pathway. The administration of Flt3 ligand (Flt3-L), a growth factor that stimulates the proliferation of hematopoietic cells, to neonates before RSV-infection, resulted in increased lung DC number, and reconditioned the IFN<sub>1</sub> pathway upon RSV neonatal infection. Besides, neonates treated with Flt3-L were protected against exacerbated airway disease upon adult reexposure to RSV. This was associated with a reorientation of RSV-specific responses toward Th1-mediated immunity. Thus, the poor lung DCs and IFN<sub>1</sub> responses to RSV in neonates may be partly responsible for the deleterious long-term consequences revealed upon adult reexposure to RSV, which could be prevented by Flt3-L treatment. These results open new perspectives for developing neonatal immuno-modulating strategies to reduce the burden of bronchiolitis.

**Keywords:** Dendritic cells · Flt3-L · Interferon · Lung · Neonate · Respiratory syncytial virus



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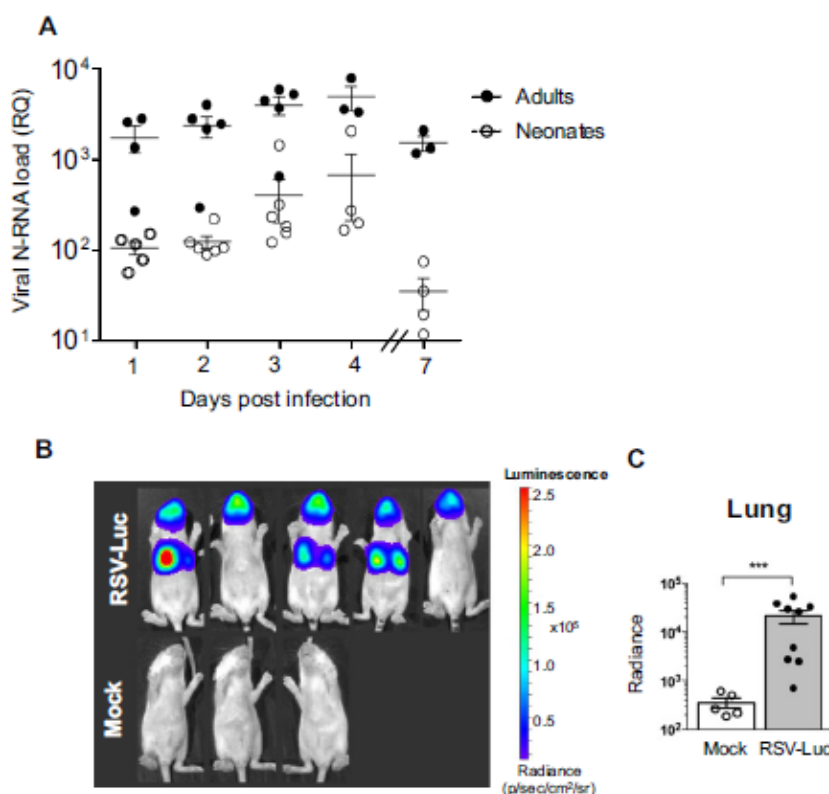
## Introduction

The respiratory syncytial virus (RSV) is the leading cause of severe bronchiolitis in infants worldwide [1, 2]. However, only 2–3% children, mostly before 6 months-of-age, will experience an acute lower respiratory disease requiring hospitalization [3]. These variations in disease severity seem mostly related to poly-

morphisms in innate immunity genes, including many that are part of the IFN system [4]. Moreover, infants with severe bronchiolitis have elevated IL-4/IFN- $\gamma$  ratio in nasal washes, which suggest that disease severity is associated with an excess of type 2 immune responses [5]. However, the immunological pathways leading to Th2-driven acute bronchiolitis have not been elucidated and the links with increased risk of asthma later in childhood are still debated [6, 7].

A neonatal mouse model of RSV infection was developed to obtain a better picture of immunity to RSV in infants and its long-term impact on airway disease [8, 9]. When BALB/c

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**Figure 1.** RSV replication in the lung of neonatal mice. (A) The viral N-RNA load from RSV-infected BALB/c mice ( $n = 24$  neonates,  $n = 20$  adults), expressed as RQ, was measured by q-RT-PCR. RQ was calculated from the Ct (cycle threshold) of N RNA normalized to Ct of HPRT mRNA ( $\Delta Ct$ ), relative to the mean of 1 dpi infected neonates ( $RQ = 100 \times 2^{-\Delta\Delta Ct}$ , with  $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \text{mean } \Delta Ct_{\text{pups at 1 dpi}}$ ). Data are mean  $\pm$  SEM. The entire kinetic was done once, and critical time points (2, 3, and 4 dpi) were reproduced in four independent experiments. (B) Nine mice neonates were infected with RSV-Luc and in vivo bioluminescence intensity was measured 2 dpi with IVIS imaging system. A digital false-color photon emission image of the mouse was generated (scale bar shown on the picture). The picture represents one of two experiments with similar data. (C) Luciferase activity in BALB/c mice neonates was measured in whole lung area by quantification of photon emission (radiance in photon/sec/cm<sup>2</sup>/sr). Data are mean  $\pm$  SEM from  $n = 5$ –9 mice and are pooled from two independent experiments.

mice are infected with RSV before 1 week of age, they mount poor Th1 responses characterized by little antiviral CD8 T cells producing IFN- $\gamma$  [10]. Upon reexposure, adult mice initially infected as neonates display greater Th2 responses associated with aggravated airway inflammation (eosinophilia and mucus hyperproduction) [8].

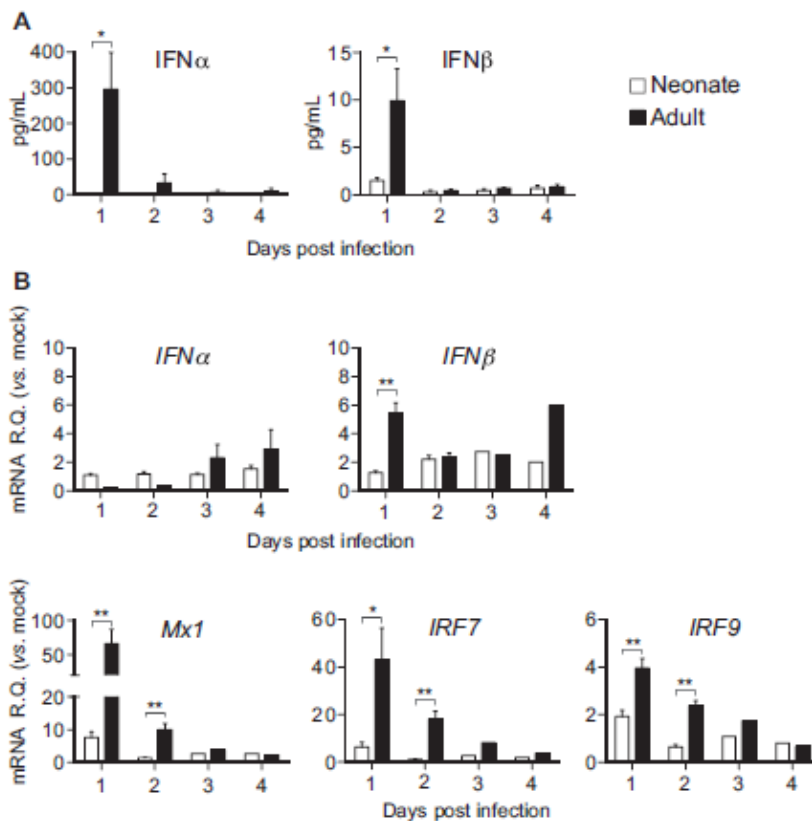
Yet little is known about how innate pulmonary immunity matures with age and the factors influencing it. We recently showed that lungs of neonate mice have a marked deficit of DCs, especially plasmacytoid DC (pDCs) and CD103<sup>+</sup> conventional DC (cDCs) with a profile of cytokines and transcriptional factors suggesting a Th2 orientation at steady state [11]. Here, we investigated the early events occurring in the lung upon RSV neonatal infection. We focused on IFN $\beta$  and DC-related pathways that we found to be severely impaired in neonates. Flt3 ligand (Flt3-L) is a hematopoietic growth factor, that triggers expansion of cDCs and pDCs in human cord blood and strongly promotes IFN- $\alpha$  production by pDCs in response to viral exposure (reviewed in [12]). Thus, for the first time in the context of a neonatal respiratory virus infection, we explored whether a treatment with Flt3-L could augment DCs and IFN $\beta$  responses, like it does in mouse adult lungs or in neonate spleen [13, 14]. Importantly, we could also evaluate the long-term impact of Flt3-L treatment on preventing airway-exacerbated disease upon adult reexposure to RSV.

## Results

### RSV infection in neonates failed to engage the IFN $\beta$ signaling pathway

We investigated whether neonatal lungs were fully permissive to RSV replication. Neonate (6 days) and adult (6 weeks) mice were infected with RSV-A2, or mock-infected. Although, RSV N-RNA loads in neonates were lower than in adults at all-time points, they followed a rather similar pattern (Fig. 1A). To establish that neonatal RSV infection was well productive in neonatal lungs, we used a recombinant strain of RSV expressing Luciferase (RSV-Luc) [15]. The strong luminescence signal recorded in neonates at 2 dpi both in snout and lungs showed that RSV replicated productively in neonates (Fig. 1B) like we showed in adults [15]. The quantification of the luminescence signal in lungs showed that the level of infection could vary but was highly significant compared with Mock-treated neonates (Fig. 1C).

In agreement with a recent study showing limited IFN $\beta$  responses in neonates [16], IFN- $\alpha$  and IFN- $\beta$  proteins were not detectable in BAL of neonates while both were found in adults at significant levels at 1 dpi and declined after 2 dpi (Fig. 2A). Accordingly, IFN- $\beta$  mRNA at 1 dpi, *Mx1*, *IRF7*, and *IRF9* mRNA at 1–2 dpi were found strongly upregulated in adult lungs in contrast to their low levels in neonate lungs (Fig. 2B). All transcripts were



**Figure 2.** IFN $\beta$  signaling pathways were upregulated by RSV infection in adults but not in neonates. (A) IFN- $\alpha$  and IFN- $\beta$  secretions from RSV-infected BALB/c mice were measured in BAL by ELISA. (B) RNA was extracted from the lungs of 58 neonates (30 RSV- and 28 Mock-infected) and 50 mice (25 RSV- and 25 Mock-infected). RNA collected at indicated dpi were reverse transcribed and the level of expression of some main genes of the IFN pathways was evaluated by q-RT-PCR. Data were normalized to HPRT mRNA as described in Fig. 1 and expressed as RQ (RQ =  $2^{-\Delta\Delta Ct}$ , with  $\Delta\Delta Ct = \Delta Ct \text{ sample} - \text{mean } \Delta Ct \text{ age-related Mock}$ ). (A and B) Data are mean  $\pm$  SEM from  $n = 5-6$  mice and represent one of two experiments with similar data.

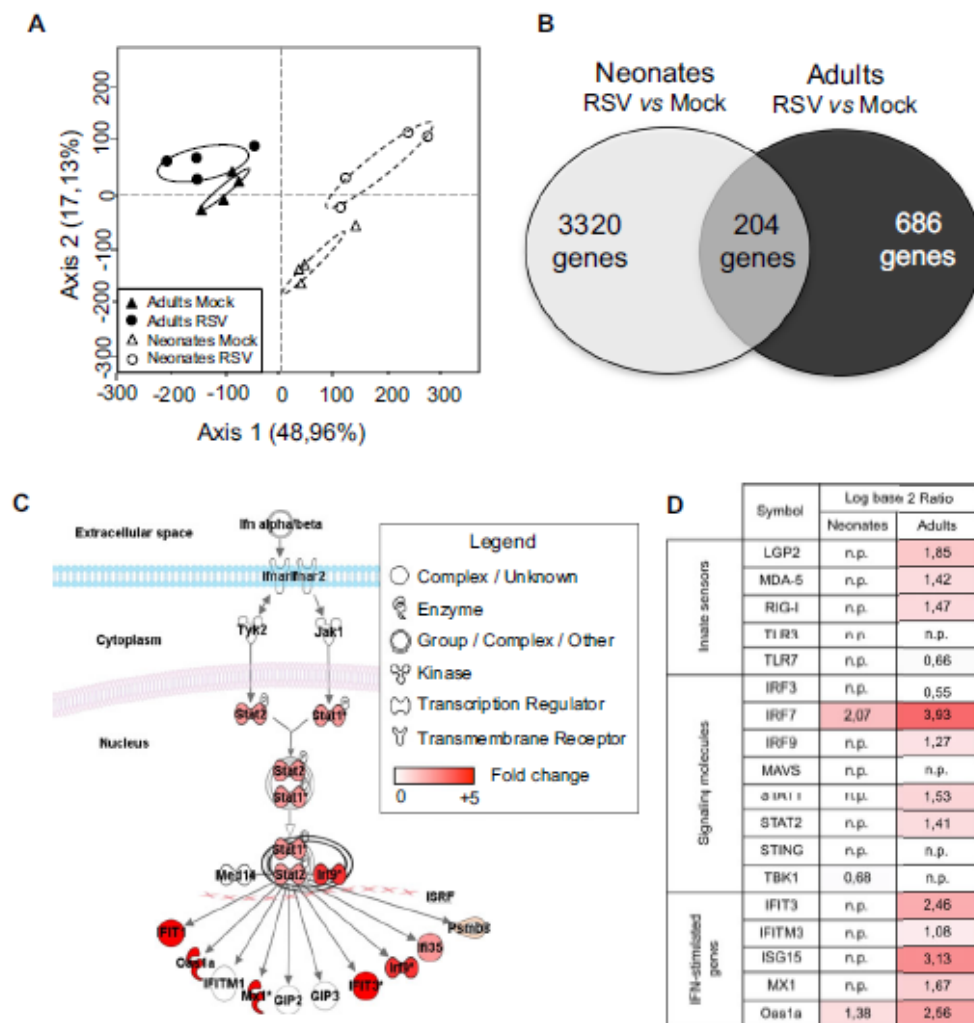
detected at same basal levels in mock-treated neonates and adults (not shown).

To gain a complete picture of the IFN-signaling pathway and of other related pathways in RSV-infected neonates, the lung transcriptomic signature of neonatal and adult mice was established at 2 dpi using whole mouse genome microarrays. The different treatments (RSV-infected versus mock-infected adults and neonates) were significantly discriminated in an unsupervised Principal Component Analysis (Fig. 3A). The first axis (explaining 48.96% of the variance) separated our samples according to age, and the second axis (17.13%) discriminated RSV-infected and Mock-treated animals within each age group. When comparing gene expression in RSV versus mock-treated mice, we found 3524 deregulated genes (2867 up, 657 downregulated) upon neonatal infection whereas only 890 genes were deregulated (759 up, 131 downregulated) upon adult infection. Only a small proportion of deregulated genes was common to adults and neonates as shown using a Venn diagram (Fig. 3B). We used ingenuity pathway analysis (IPA) to explore the list of differentially expressed genes upon RSV infection. Within the IPA pathway related to IFN $\beta$  signaling (Fig. 3C), most genes were found strongly upregulated in adults but not in neonates. This was illustrated by the increase of ISG and innate sensor genes (Fig. 3D). Thus, not only did neonates have limited IFN $\beta$  production but all the IFN $\beta$  signaling cascade was altered.

### Neonates had weak DCs recruitment in the lung after RSV infection

IFN $\beta$  is also a major driver for innate responses leading to immune cells recruitment in lungs [17]. In fact, our IPA analysis showed that DC-involving pathways were differently mobilized in adults and neonates upon RSV infection (Fig. 4A). Since, we have reported a defect in lung DCs at steady state in neonates [11], we undertook to study lung DCs recruitment during RSV neonatal infection. pDCs were defined as MHCII<sup>med</sup> CD11c<sup>+</sup> mPDCA1<sup>+</sup> cells and cDCs as MHCII<sup>high</sup> CD11c<sup>+</sup> cells, either CD11b<sup>+</sup> CD103<sup>-</sup> (CD11b<sup>+</sup> cDCs) or CD11b<sup>-</sup> CD103<sup>+</sup> (CD103<sup>+</sup> cDCs) [11]. pDCs were rapidly recruited in infected adult lungs, peaked at 3 dpi and decreased at 4 dpi (7.3-fold increase versus Mock at 3 dpi, Fig. 4B). The number of pDCs in lungs from Mock and RSV-infected neonates was much lower than in adults. It slowly increased from 3 to 7 dpi most likely due to increasing age as there was no significant difference between mock and infected pups, except at 4 dpi (2.2-fold increase in infected lungs versus Mock). Both subsets of cDCs displayed a similar kinetic in infected adult lungs: they increased significantly at 1 and/or 2 dpi, peaked at 4 dpi (5.5- and 4.1-fold increases versus Mock for CD103<sup>+</sup> and CD11b<sup>+</sup> subsets, respectively) and decreased at 7 dpi (Fig. 4C and D). In lungs of RSV-infected neonates, the kinetic seemed delayed compared with adults: CD11b<sup>+</sup> cDCs weakly but significantly





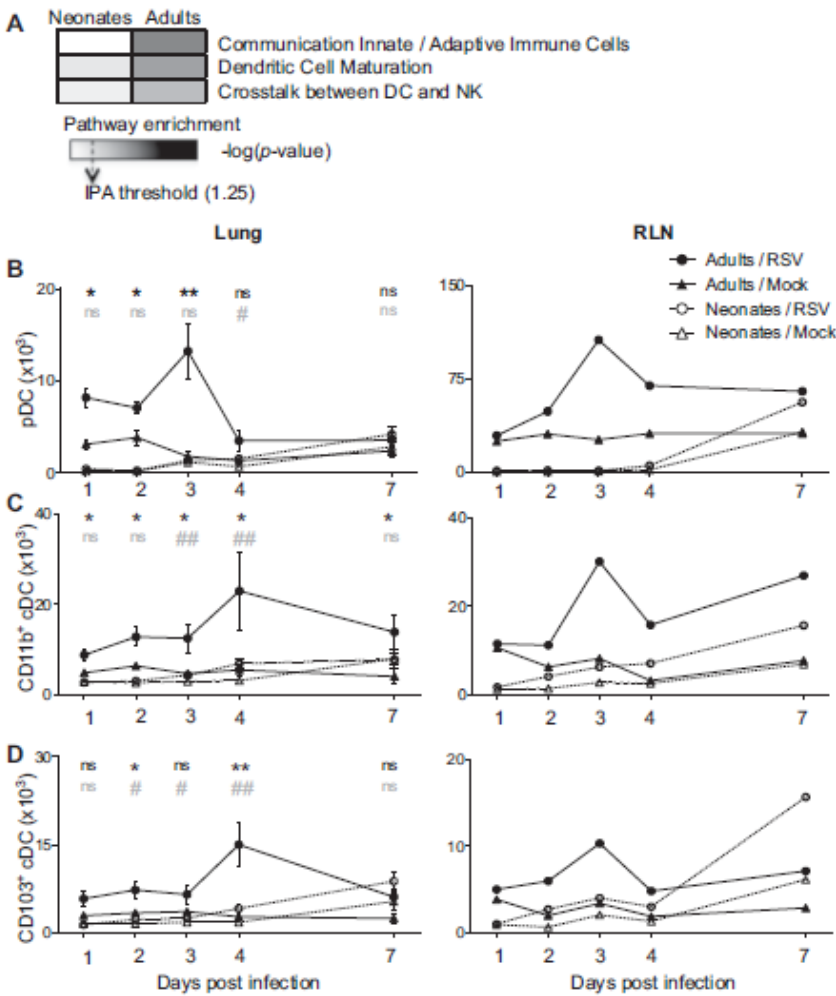
**Figure 3.** Transcriptomic analysis revealed major differences in early responses to RSV infection according to age. Neonate (6 days) or adult (6 weeks) BALB/c mice were infected with RSV. Controls were Mock-infected with cell culture supernatants. (A) Principal components analysis (PCA) on normalized genes from four mice per group was performed using the FactoMineR package. The circles delimit the inclusion in a group for a confidence level of 95%. (B) Venn diagram showing the distribution of deregulated genes relative to mock-infected mice at both ages. Statistical analysis was performed using a linear integrated model for microarray analysis (limma). All *p*-values were corrected with Benjamini Hochberg. (Cut-off: adjusted *p*-value < 0.05 and fold change > 1.5). (C) IFN pathway overview in RSV-infected adult mice. The pathway was drawn using Path Designer in Ingenuity Pathway Analysis (IPA) software. Upregulated genes are shown in red. (D) Heat map of selected genes related to the IFN pathway in the list of regulated genes (upregulated genes are shown in red) of infected neonates or adults based on analysis using Ingenuity Pathway Analysis software (IPA). Data are expressed in Log base 2 ratio. n.p. (not present) means that this gene is not in the corresponding differentially expressed gene list (3B). Microarray data (2 dpi RNA) were normalized to the global median (*n* = 4 per group).

increased at 3 and 4 dpi, and CD103<sup>+</sup> cDCs at 2, 3, and 4 dpi (4 dpi, 2.3- and 2.2-fold increases versus Mock for CD103<sup>+</sup> and CD11b<sup>+</sup> subsets, respectively). We studied the recruitment of these DC subsets in respiratory draining lymph node (RLN) and we observed a peak of cDCs and pDCs at 3 dpi in RSV-infected adult mice whereas in neonate mice, DC numbers stayed low, increasing slowly at 4–7 dpi (Fig. 4B, C, and D, right). Interestingly, *Flt3* mRNA receptor was detectable in neonatal lungs as early as 7 days of age and its level of expression significantly increased upon RSV infection (7 dpi) (Supporting Information Fig. 1).

In summary, neonates have a late and rather small DCs recruitment to their lungs and RLN upon RSV infection, which may influence subsequent priming of immune responses.

### Flt3-L treatment of neonates augmented IFN<sub>γ</sub> responses, lung DCs and CD8 T cells mobilization in lungs

We attempted to boost DC numbers by Flt3-L administration in pups from 2 to 6 days of age. At 6 days, pups in both groups were

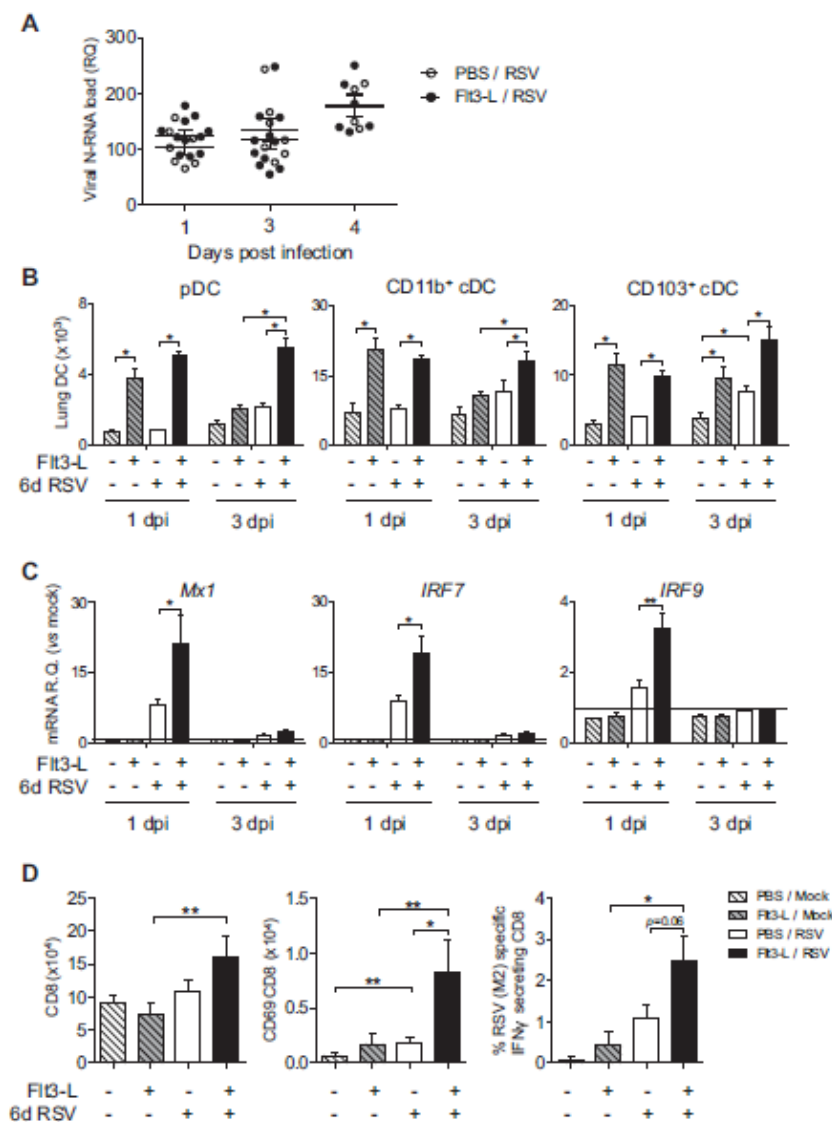


**Figure 4.** Kinetics of lung and RLN DCs recruitment in adults and neonates following RSV infection. Neonate (6 days) or adult (6 weeks) BALB/c mice were infected with RSV. Controls were Mock-infected with cell culture supernatants. (A) Heat map of IPA DC pathway enrichment from microarray data, analyzed as described in Fig. 3. (B, C, D) RLN and lung cells, isolated at different time points from RSV or mock infected neonatal or adult BALB/c mice, were labeled with anti-CD11c, -MHCII (I-A/I-E), -CD11b, and -CD103 or -mPDCA1 mAb or matching isotype controls. (B) pDCs were identified as CD11c<sup>+</sup> MHCII<sup>high</sup> cells and analyzed for their expression of CD11b and CD103, defining the two major subsets of cDCs: CD11b<sup>+</sup> CD103<sup>-</sup> (CD11b<sup>+</sup>) (C) and CD11b<sup>-</sup> CD103<sup>+</sup> (CD103<sup>+</sup>) (D). Lung data are mean  $\pm$  SEM from  $n = 5-6$  mice per group. Statistics compare Neonates, Mock to RSV (gray hash symbols), and Adults, Mock to RSV (black stars). RLN data represent a pool value of  $n = 5-6$  mice per group. The entire time course was done once, and critical time points (1, 3, and 4 dpi) were reproduced twice in independent experiments. All data were acquired from  $\geq 2 \times 10^6$  total cells.

infected with RSV or Mock-treated. Similar amounts of viral RNA were detected in the lungs of PBS and Flt3-L treated, infected pups, showing that Flt3-L treatment had no effect on virus replication (Fig. 5A). Flt3-L treatment successfully increased the numbers of all subsets of DCs at 1 dpi in Mock-infected pups ( $p < 0.05$ , Flt3-L/Mock versus PBS/Mock) but the numbers of pDCs and CD11b<sup>+</sup> cDCs decreased 2 days later (Fig. 5B). Conversely, Flt3-L treatment allowed a sustained recruitment of pDCs and cDCs in the lung upon RSV infection ( $p < 0.05$ , Flt3-L/RSV versus PBS/RSV at 1 and 3 dpi), with cDC numbers reaching adult values at 3 dpi and pDC numbers, half adult values (Fig. 4A, B, and C). The surface expression of maturation markers like CD86 was not found augmented on cDC subsets (Supporting Information Fig. 2). We further assessed the effects of Flt3-L treatment on the activation of ISG expression (Fig. 5C). Flt3-L treatment did not change the basal level of *Mx1*, *IRF7*, and *IRF9* transcripts in mock-treated neonates, but it significantly increased the level of expression of *Mx1*, *IRF7*, and *IRF9* genes in RSV-infected neonates (at 1 dpi, PBS/RSV versus Flt3-L/RSV;  $p < 0.05$  for *Mx1* and *IRF7*,  $p < 0.01$  for *IRF9*). Thus, Flt3-L treatment during the neonatal period

increased the number of all lung DC subsets, and restored IFN $\gamma$  pathways.

Tregoning et al. showed that upon RSV infection, neonates have less T CD4 and T CD8 activated in lungs than adults and that neonatal lung CD8 T cells were deficient for IFN $\gamma$  production [10]. Thus, we investigated the outcome of Flt3-L treatment on T cells activation upon neonatal RSV infection by monitoring the early activation marker CD69. Flt3-L treatment strongly augmented the number of CD8<sup>+</sup> CD69<sup>+</sup> T cells at 7 dpi in lungs (Fig. 5D;  $p < 0.05$ , Flt3-L/RSV versus PBS/RSV; see Supporting Information Fig. 3A for gating strategy) but not of CD4<sup>+</sup> CD69<sup>+</sup> T cells (Supporting Information Fig. 3B). The frequency of RSV (M2<sub>82-90</sub>) peptide-specific IFN $\gamma$ -producing CD8 T cells was also augmented upon infection of Flt3-L-treated neonates but not significantly ( $p = 0.06$ ) (Fig. 5D). Likewise, the level of IFN $\gamma$  in BAL was not augmented by Flt3-L treatment (Supporting Information Fig. 3C). We also measured the levels of IL-5 and IL-10 in BAL and did not detect any cytokine production above the detection limit of our assay. Therefore, the outcome of Flt3-L treatment on T-cell responses to neonatal RSV infection was mostly noticeable on T CD8 activation.



**Figure 5.** Flt3-L treatment prior to neonatal RSV infection improved IFN $\gamma$  signaling pathways, DCs recruitment and CD8 T cells activation in lungs. BALB/c mice (2–6 days of age) received daily a subcutaneous administration of 1  $\mu$ g mouse recombinant Flt3-L or 20  $\mu$ L of PBS. At 6 days, BALB/c mice were infected by i.n. administration of 10  $\mu$ L of RSV (10<sup>6</sup> PFU) or Mock-infected and sacrificed at day 1, 3, or 4 pi. (A–C) Graphs represent one of three experiments with similar data. (A) Viral N-RNA load in lungs was determined by q-RT-PCR as described in Fig. 1. RQ values are expressed relatively to 1 dpi PBS-treated RSV-infected pups. Data are represented individually with corresponding lines representing mean  $\pm$  SEM (n = 5–11 mice). (B) Lung cells isolated at 1 and 3 dpi were labeled as described in Fig. 4. (C) q-RT-PCR data were normalized as described in Fig. 2 and expressed as RQ (RQ = 2<sup>- $\Delta\Delta$ Ct</sup>, with  $\Delta\Delta$ Ct =  $\Delta$ Ct sample – mean  $\Delta$ Ct age-matched Mock). The dotted line (RQ = 1) represents the expression level of Mock-infected mice. (D) Neonate mice were killed 7 days after initial RSV infection. Lung cells were labeled with anti-CD3, -CD8, or -CD69 mAb (or matching isotype controls) or stimulated 4 h with RSV M2<sub>82-90</sub> peptide and stained for T cells, then for intracellular IFN- $\gamma$ . T lymphocytes were studied by gating on CD45<sup>+</sup> CD3<sup>+</sup> CD8<sup>+</sup> cells and analyzed for their expression of CD69 or IFN- $\gamma$ , defining an activated phenotype. Data were acquired from 2  $\times$  10<sup>6</sup> cells with a FACScalibur or FACSFortessa and analyzed with the FlowJo Software v7.5. One of two experiments with similar data is shown. (B–D) Data are shown as mean  $\pm$  SEM of n = 4–9 mice per time point.

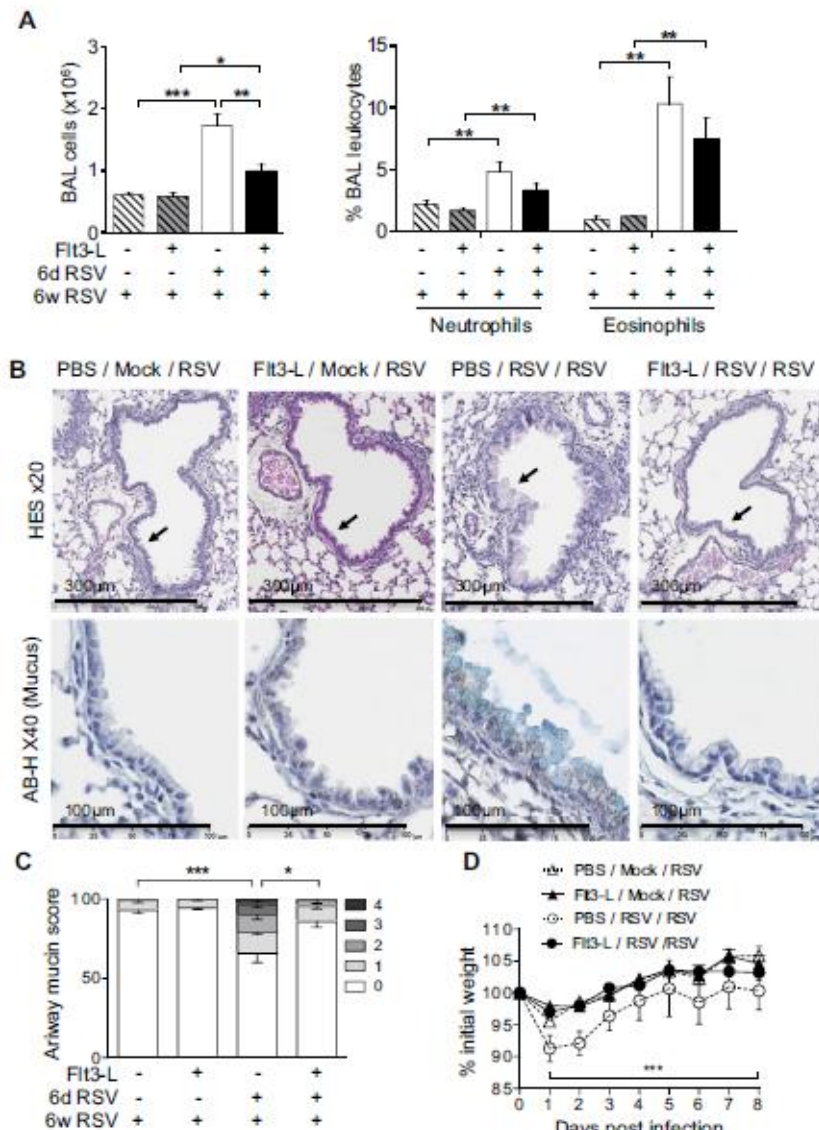
### Flt3-L treatment of neonates reduced airway inflammation upon reexposure to RSV at adult age

To explore the long-term effect of the neonatal Flt3-L treatment, we reexposed the mice to RSV when adults and monitored their lung inflammation. At 8 dpi, the total number of BAL cells was significantly increased when mice had been infected first with RSV as neonates and reexposed when adults (PBS/RSV/RSV) compared with an adult infection (PBS/Mock/RSV) (Fig. 6A,  $p < 0.01$ ). The total number of BAL cells recovered from Flt3-L/RSV/RSV-treated mice was significantly lower than in the group PBS/RSV/RSV ( $p < 0.01$ ) (Fig. 6A). However, similar percentages of neutrophils and eosinophils were found in BAL of Flt3-L/RSV/RSV and PBS/RSV/RSV groups, and the proportions of these granulocytes were significantly higher than after a first infection with RSV at adult age (PBS or Flt3-L/Mock/RSV)

(Fig. 6A,  $p < 0.01$ ). Yet, the total influx of eosinophils and neutrophils was reduced when neonates were treated with Flt3-L before RSV infection.

We also performed histological analysis of lung tissue. In comparison with adults infected with RSV for the first time and as expected from the literature [18], lungs from PBS/RSV/RSV mice showed numerous peribronchiolar and perivascular immune infiltrates with abundant mucus production by goblet cells (Fig. 6B). This inflammatory pattern was markedly reduced in lungs from the Flt3-L/RSV/RSV group (Fig. 6B). Accordingly, the airway mucin-score was high in the PBS/RSV/RSV group and significantly reduced in the Flt3-L/RSV/RSV group (Fig. 6C;  $p < 0.05$ ). As expected, mice primary infected as pups and reexposed to RSV when adults (PBS/RSV/RSV) presented delayed weight gain (Fig. 6D,  $p < 0.001$  versus all other groups). Flt3-L treatment of neonatal mice prevented this delayed growth upon reexposure to





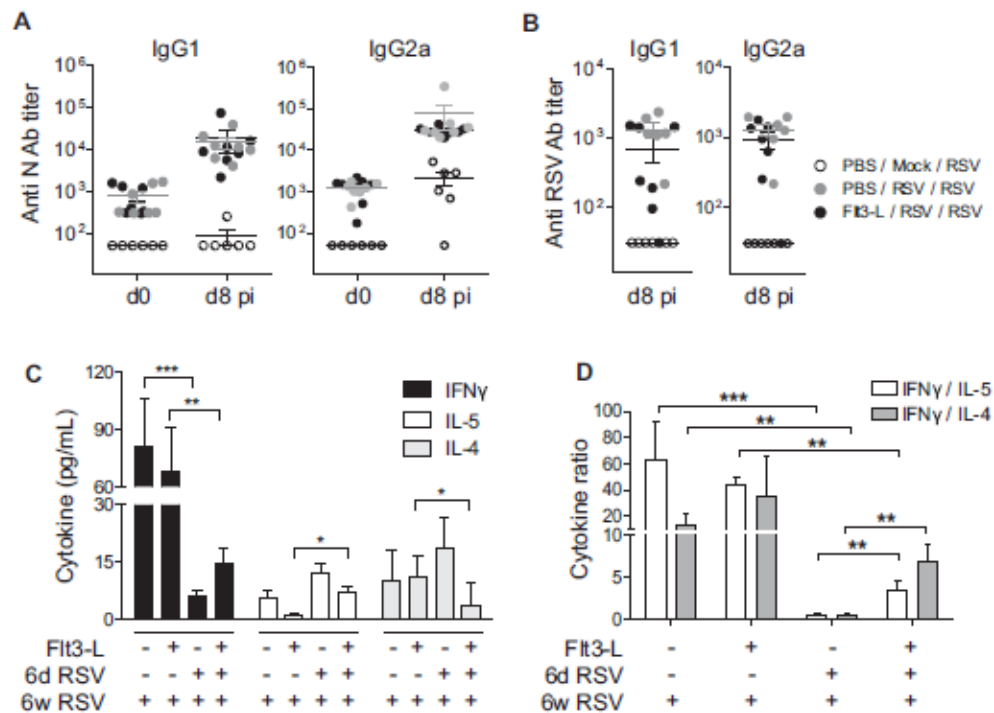
**Figure 6.** Flt3-L treatment prior to neonatal RSV infection protected against pathology upon adult reexposure. BALB/c mice were treated as described in Fig. 5. At 6 weeks all mice were infected with 50  $\mu$ L of RSV (5.10<sup>6</sup> PFU) and sacrificed at 8 dpi. One of three experiments with similar data is represented. Each group represents n = 6-10 mice. (A) BAL cells were enumerated, cytocentrifuged, and stained with May-Gründwald-Giemsa. Neutrophils and eosinophils were enumerated and expressed as % of total BAL cells. Data are mean  $\pm$  SEM. (B) Lungs were fixed, embedded in paraffin and sectioned at 5  $\mu$ m. Lung sections were stained with hematoxylin-eosin-saffron (HES) or alcian blue-hematoxylin (AB-H) and photographed using a Nanoscope. One representative section per group is shown. AB-H line shows higher power images of the area indicated by arrow in HES line (scale bar represents 300  $\mu$ m for HES and 100  $\mu$ m for AB-H). (C) Individual airways were scored for goblet cells and mucins (AB-H staining) according to the following scale: 0 = 0%, 1  $\leq$  25%, 2  $\leq$  50%, 3  $\leq$  75%, and 4 = 100% of AB-H positive cells (according to [26]). All airways involved in the lung sections were scored (171  $\leq$  n  $\leq$  215 per group). Data represent the mean  $\pm$  SEM of mucin score. (D) Mice were weighed daily upon RSV infection. The growth curves are mean  $\pm$  SEM of individual weight (% of initial weight at day 0). Tukey's multiple comparison test, repeated measures one way ANOVA, was used for comparison of growth curves between 1 and 8 dpi.

the virus (Flt3-L/RSV/RSV) as their weight curve was not significantly different from that of control adult mice infected for the first time. Thus, neonatal Flt3-L treatment protected mice from exacerbated disease upon reexposure at adult age.

**Flt3-L treatment of neonates improves Th1 responses revealed upon RSV reexposure**

We next investigated whether neonatal Flt3-L treatment would impact the Th2 response expected for adults upon RSV reexposure [8]. Control adult mice infected with RSV for the first time (PBS/Mock/RSV) produced mainly IgG2a Ab against the nucleoprotein N at 8 dpi, indicative of Th1-dependent immunity (Fig. 7A). In contrast, mice infected with RSV as neonate and reex-

posed when adult, produced high titers of IgG2a and IgG1 Ab to N (Fig. 7A) or whole RSV antigens (Fig. 7B), revealing that the neonatal infection had primed both type 1 and type 2 immune responses, irrespective of Flt3-L treatment. BAL collected 8 dpi after a first RSV infection of adult mice contained high levels of IFN- $\gamma$  and low levels of IL-5 and IL-4 (Fig. 7C). By comparison, BAL collected from mice infected with RSV as neonate and reexposed when adult contained less IFN- $\gamma$  (Fig. 7C,  $p < 0.01$ ) and more IL-5 and IL-4 (Fig. 7C). Flt3-L treatment before the neonatal RSV infection tended to augment the level of IFN- $\gamma$  and to reduce the levels of IL-5 and IL-4 upon adult RSV reexposure (Fig. 7C). Importantly, the ratio of both IFN- $\gamma$ /IL-5 and IFN- $\gamma$ /IL-4 were increased in Flt3-L-treated neonate mice (Fig. 7D,  $p < 0.01$ ). Thus, Flt3-L treatment reoriented to some extent neonatal immune responses toward Th1 responses, revealed upon adult reexposure.



**Figure 7.** FIt3-L treatment changed Th1/Th2 balance and T-cell activation upon adult or neonatal RSV infection. BALB/c mice treated as described in Fig. 6 were bled before (0 dpi) and after (8 dpi) an RSV infection or reexposure at adult age. Individual mouse sera were assayed for specific Ab (IgG1, IgG2a) against RSV N protein (A) or RSV lysates (B) by ELISA. Individual titers and the mean titer  $\pm$  SEM are shown. (C) At 6 dpi, IL-5, IL-4, and IFN- $\gamma$  secretions were measured in BAL by ELISA and the ratio of IFN- $\gamma$ /IL-4 or IL-5 (D) are calculated (mean  $\pm$  SEM from  $n = 6$ –10 mice per group). One of three experiments with similar data is shown.

## Discussion

Our study addresses the IFN $\gamma$  pathway and the mobilization of DCs in lungs, two key innate components in the setting of neonatal antiviral immunity. FIt3-L administration to neonates prior to infection was evaluated for its capacity to promote both responses and to modify the neonatal pathological imprinting.

RSV infection in adult was characterized by an early transient release of IFN $\alpha/\beta$  in BAL and a subsequent increase in *Mx1*, *IRF7*, and *IRF9* transcripts at 1 and 2 dpi, in agreement with previous studies [17, 19]. In addition to their antiviral effects, IFN $\gamma$  act as proinflammatory mediators in RSV disease. Indeed all IFN subtypes (I, II, and III), ISG and proinflammatory cytokines (e.g. IL-6, IL-1 $\beta$ , TNF $\alpha$ ) normally released in the lungs upon RSV infection are missing in IFNAR1-deficient mice [17].

In contrast, IFN $\gamma$  signaling pathway was weakly engaged in neonates upon infection (lack of IFN $\alpha/\beta$ ; weak and transient upregulation at 1 dpi of *Mx1*, *IRF7*, and *IRF9* transcripts). Our microarray analysis confirmed that the IFN $\gamma$  signaling pathway was fully engaged in adults upon RSV infection, but not in neonates for whom the whole downstream ISG cascade was altered. The limited release of IFN $\gamma$  in neonatal lungs upon RSV infection was already reported, but without an investigation of the whole pathway [16]. In this study, Cormier et al. make the hypothesis that the weak IFN $\gamma$  response is due to limited pDCs recruitment to the

lungs upon RSV infection [16]. However, alveolar macrophages were recently identified as the prominent source of IFN $\gamma$  upon RSV infection in mice, with only a limited contribution from pDCs [20]. IFN $\gamma$ -producing alveolar macrophages are driving the recruitment of monocytes-derived inflammatory cells, necessary and sufficient to control RSV infection and RSV-induced pathology [20]. Whether pDCs or/and alveolar macrophages are the main defective source of IFN $\gamma$  in neonatal lungs upon RSV infection is a major issue that remains to be solved.

In fact, we observed that most pathways related to DCs were more efficiently mobilized in adults than in neonates upon RSV infection, and both pDC and cDC subsets were poorly represented in the lungs and RLN of infected neonates. The CD103<sup>+</sup> cDC subset was recently shown in neonate mice to replicate RSV and to migrate to RLN albeit with poor costimulatory properties and this influenced the repertoire of RSV-specific CD8 T cells [21].

In adult mice, Smit et al. used FIt3-L treatment to expand lung CD11b<sup>+</sup> cDCs and pDCs, and showed that it attenuates RSV-induced pathology in a pDC-dependent manner [22]. Using similar experimental settings in neonates, we increased cDC and pDC numbers in lungs, transiently in mock-infected neonates and steadily upon RSV infection, without affecting virus replication. FIt3-L treatment restored ISG upregulation in neonatal lungs upon RSV infection. Yet we could neither detect IFN $\gamma$  gene upregulation, nor increased IFN $\alpha/\beta$  secretion in BAL (data not shown).



Interestingly, Flt3-L significantly augmented the priming of lung CD8 T cells (shown by CD69 expression and RSV M2 peptide-specific IFN- $\gamma$  production at 7 dpi) upon neonatal infection with RSV. Thus our data add to the demonstration of a relationship between weak DCs neonatal responses and poor CD8 T cells priming [21].

Most importantly, neonatal Flt3-L treatment protected mice from exacerbated pathology upon adult reexposure, as shown by improved weight gain, less inflammatory cellular infiltrate in BAL and less mucus deposition in airways. The immune parameters indicative of a Th2 pathological imprinting were all reduced (reduction of eosinophilia and mucus production, increased ratio of Th1/Th2 cytokines).

In the present study, we have chosen to use RSV grown in cell culture. In order to control for non specific effects induced by the presence of serum proteins in our RSV batch, we have treated the Mock control groups with Hep2 supernatant. We remain aware that the T-cell response to serum proteins could be stronger in the RSV groups than in the Mock groups due to an adjuvant effect of the viral infection.

Other studies have attempted to modulate the consequences of early life RSV infection on later lung health. Thus, exposure to CpG prior to a neonatal RSV infection increases MHCII and CD80 expression on CD11c<sup>+</sup> cells, IFN- $\gamma$  production by NK cells and is protective against enhanced disease upon adult reexposure, leading to the conclusion that the protective effects of CpG are mediated by DCs and NK cells [23]. However, in our hands, Flt3-L treatment did not significantly increase NK cells in neonatal lung (data not shown). In the study by Cormier et al. neonatal treatment with either recombinant IFN $\alpha$  or passive transfer of adult pDCs considerably decreased Th2 responses and airway disease during RSV reinfection [16]. However both these treatments directly diminished RSV replication in neonatal lungs and may have limited this way the priming of pathogenic anti-RSV responses. In contrast Flt3-L did not modify viral load in lungs and boosted endogenous lung DCs and IFN $\gamma$  in the neonatal environment. This was sufficient to reduce lung pathology upon reexposure and to partly restore Th1 priming. In fact, the long-term effect of Flt3-L treatment in our model of virus-driven airway disease is reminiscent of the protective effect of Flt3-L in mouse model of allergic responses [24].

Further studies will be necessary to ascribe specific roles to pDCs, cDCs, and IFN $\gamma$  in driving neonatal immune responses toward protective or pathological responses revealed by adult reexposure to RSV. Our findings open the way to new immunostimulating strategies to reduce the burden of bronchiolitis and its detrimental consequences in infants.

## Materials and methods

### Animals and infection

Animal experiments were approved by the COMETHEA ethics committee under authorization number 11–035. BALB/c mice

purchased from Janvier (Le Genest, St. Isle, France) were bred and housed under FELASA SPF conditions in our animal facilities (IERP, INRA, Jouy-en-Josas). Neonate (6 days) or adult (6 weeks) mice received respectively 10 or 50  $\mu$ L of RSV-A2 ( $10^8$  PFU/mL), recombinant RSV expressing luciferase (RSV-luc,  $1.75 \times 10^6$  PFU/mL, [15]) or Hep2 cell culture supernatant (Mock-infection) by intranasal instillation. Mice were anesthetized by i.p. injection of ketamine and xylazine. In some experiments, 2 days old pups received 5 daily subcutaneous injections of 1  $\mu$ g of mouse Flt3-L (eBioscience) or diluent LPS-free PBS (Lonza) under a volume of 20  $\mu$ L. At 6 days, some pups were infected with RSV while others were left uninfected. Some pups were killed between 1–7 dpi whereas the others were kept until 6 weeks of age when they were all infected with RSV.

### Sample collection

Mice were bled by retro-orbital puncture and killed by cervical dislocation. The left bronchus was clamped and bronchoalveolar lavage (BAL) was performed on the right lobes with PBS 1 mM EDTA as described [11, 25]. BAL supernatants were stored frozen at  $-20^{\circ}\text{C}$  and BAL cells were cytocentrifuged (Cytospin 5) on microscope slides (Superfrost), then stained with May-Grünwald and Giemsa. The right lung, perfused with PBS, and the respiratory lymph nodes (RLN) (cervical, maxillary, and mediastinal LN) were treated with 1 mg/mL collagenase D and 0.5 mg/mL DNase I for cell isolation. The left lobe of the lung was kept frozen at  $-80^{\circ}\text{C}$  until processed for RNA extraction. Alternatively, for histological examination, the lungs were fixed with 4% paraformaldehyde and embedded in paraffin. Five micrometers sections were stained with hematoxylin/eosin/saffron or alcian blue and photographed using a NanozoomerDigitalPathology.view software (Hamamatsu).

### Viral N-RNA load and gene expression by qRT-PCR

Total RNA was extracted from lung homogenate using RNA minikit columns (Qiagen) and reverse transcribed using random primers and M-MLV Reverse Transcriptase (SuperScript II, Invitrogen) according to the manufacturer's instructions. The primers (Sigma-Aldrich) used are listed in Supporting Information Table 1. qRT-PCR was run in triplicate for each gene using the MasterCycler<sup>®</sup> realplex (Eppendorf) and SYBRGreen PCR Master Mix (Eurogentec) and data analyzed with the Realplex software (Eppendorf) to determine the cycle threshold (Ct) values.

### Bioluminescence measurements

Photon emission was counted in the lungs of RSV-Luc or Mock infected pups using the IVIS system (Xenogen Biosciences) as described for adult mice [15]. Briefly, mice received 50  $\mu$ L of luciferin (30 mg/mL, Perking Elmer) i.p. and luciferase activity was measured with Living Image software (version 4.0, Caliper Life Sciences). Bioluminescence images were acquired for 1 min

with  $f/\text{stop} = 1$  and binning = 8. A digital false-color photon emission image of the mouse was generated, and photons were counted within a constant region of interest corresponding to the surface of the chest encompassing the whole lung area. Photon emission was measured as radiance in  $\text{photon sec}^{-1} \text{cm}^{-2} \text{sr}^{-1}$ .

### Microarray experiments

Quality of lung RNA was checked on RNA 6000 Nano Chip kit (Agilent) with an Agilent 2100 Bioanalyzer. Two hundred nanograms were amplified by linear PCR and labeled with Cy3 (LowInput QuickAmp Labeling Kit One-Color, Agilent). Each Cy3 labeled cDNA was hybridized onto murine  $4 \times 44\text{K}$  microarrays (Agilent) following the manufacturer's protocol. Agilent Feature Extraction v8.5 software was used to generate QC Reports that revealed no difference between all 16 arrays. Raw data were extracted from scanned microarray images using Feature Extraction Software v9.5 (Agilent) and normalized to the global median (interarray normalization). Statistical analyses were performed using a Linear Model for Microarray Analysis (Limma). All  $p$ -values were corrected for multiple testing using the Benjamini and Hochberg procedure. Genes were considered deregulated when the adjusted  $p$ -values were under 0.05 and their fold change superior to 1.5. For functional analysis, the gene list selected by this filter was subjected to ingenuity pathway analysis (IPA, Ingenuity Systems). The complete dataset has been deposited at the Gene Expression Omnibus database (accession number GSE42759).

### Flow cytometry

After saturation with anti-CD32/CD16, cells were incubated with mAb reactive to mPDCA1 (JF05-1C2.4.1, conjugated to FITC), MHCII (IA/IE, 2G9 or M5/114.15.2, FITC or PE), CD103 (M290 or 145-2D11, PE), CD86 (GL1, FITC) CD11b (M1/70, PerCP Cy5.5), or CD11c (HL3, Biotin or BV786). For T-cell staining, cells were incubated with mAb reactive to CD4 (L3T4 or RM4-5, FITC or PECy7), CD45.2 (104, A780), CD69 (H1.2F3, PE, or BV421), CD3 (145-2C11, PerCP Cy5.5), and CD8 (Ly2, 53–6.8, Biotin or APC). For IFN- $\gamma$  intracellular staining (XMG1.2, FITC), lung cells were stimulated 4 h with RSV M2<sub>82-90</sub> peptide (YIGSINNI, 1  $\mu\text{g}/\text{mL}$ , Anaspec inc.) in presence of Golgi Plug (BD Bioscience) before T-cell staining, fixation, and permeabilization with the BD fix and Perm reagents, and labeled with anti-IFN- $\gamma$  Ab. All mAb were purchased from BD Biosciences except for mPDCA1 (Miltenyi Biotec). APC-conjugated streptavidin (BD biosciences) was used to label biotin Ab. Data were acquired with a FACScalibur or a FACS- Fortessa (BD biosciences) and analyzed with the FlowJo Software v7.5 (Tree Star Inc.).

### Cytokine and RSV-specific Ab ELISA

Cytokines (IFN- $\alpha$  and IFN- $\beta$  (VeriKine™), IL-5, IL-4, and IFN- $\gamma$  (Ready-SET-Go, eBiosciences) were quantified by ELISA according

to the manufacturer's instructions. Individual mouse sera were assayed for specific Ab (IgG1, IgG2a) against RSV N-protein or RSV lysates by ELISA as described [25].

### Statistical analysis

Nonparametric Mann-Whitney (comparison of two groups,  $n \geq 4$ ),  $t$ -test (two groups,  $n \geq 3$ ), or ANOVA Tukeys multiple comparison test ( $> 2$  groups) were used to compare unpaired values (GraphPadPrism software). Significance is represented: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; and \*\*\*\* $p < 0.0001$ .

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**Abbreviations:** BAL: bronchoalveolar lavage · cDC: conventional DC · Ct: cycle threshold · IFN $\alpha$ : type I interferon · IPA: ingenuity pathway analysis · pDC: plasmacytoid DC · RSV: respiratory syncytial virus · RLN: respiratory draining lymph node

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## ORIGINAL ARTICLE

# Bacteria isolated from lung modulate asthma susceptibility in mice

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**Asthma is a chronic, non-curable, multifactorial disease with increasing incidence in industrial countries. This study evaluates the direct contribution of lung microbial components in allergic asthma in mice. Germ-Free and Specific-Pathogen-Free mice display similar susceptibilities to House Dust Mice-induced allergic asthma, indicating that the absence of bacteria confers no protection or increased risk to aeroallergens. In early life, allergic asthma changes the pattern of lung microbiota, and lung bacteria reciprocally modulate aeroallergen responsiveness. Primo-colonizing cultivable strains were screened for their immunoregulatory properties following their isolation from neonatal lungs. Intranasal inoculation of lung bacteria influenced the outcome of allergic asthma development: the strain CNCM I 4970 exacerbated some asthma features whereas the pro-Th1 strain CNCM I 4969 had protective effects. Thus, we confirm that appropriate bacterial lung stimuli during early life are critical for susceptibility to allergic asthma in young adults.**

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## Introduction

Allergic asthma is a worldwide problem with rising prevalence and morbidity in industrialized countries. Variable airway obstruction is a typical feature of this disease, caused by chronic eosinophilic airway inflammation, mucus overproduction, airway wall remodeling and bronchial hyperactivity. In allergic asthma, allergen-specific Th2 lymphocytes cause inflammation and control the synthesis of allergen-specific IgE, a hallmark of allergic sensitization (Kool *et al.*, 2012; Plantinga *et al.*, 2013; Just *et al.*, 2014). The cause of the asthma epidemic in the Western world is unclear, but its increased incidence has been correlated to changes in environment and gut microbiota (Fujimura and Lynch, 2015).

It has been proposed that the perturbation of early microbial stimulation in industrialized countries, due to improved hygiene, excessive antibiotherapy and dietary changes, results in an aberrant immune response to innocuous antigens later in life (Wills-Karp *et al.*, 2001; Noverr and Huffnagle, 2005;

Schuijs *et al.*, 2015). However, the gut dysbiosis observed in asthma did not obligatory mean a causal role of gut microbiota in the pathology. Recent epidemiological and clinical data (Hoskin-Parr *et al.*, 2013), as well as experimental data obtained in mouse models (Noverr *et al.*, 2004; Olszak *et al.*, 2012), support the key role of the microbial environment. Bacterial colonization of the gut during the first months of life is critical for the development and the balance of the immune system: it promotes the maturation of the epithelial mucosa (Cherbuy *et al.*, 2010; Tomas *et al.*, 2013, 2015), the establishment of immune tolerance (Gaboriau-Routhiau *et al.*, 2009; Cahenzli *et al.*, 2013) and improves stimulation of the immune system that helps to prevent and/or resolve infectious diseases (Cerf-Bensussan and Gaboriau-Routhiau, 2010; De Filippo *et al.*, 2010; Buffie and Pamer, 2013).

Healthy lungs have historically been considered to be sterile, but the description of a resident lung microbiota emerged a few years ago and broke this dogma. A bacterial community has been described in healthy human lungs (Charlson *et al.*, 2011; Erb-Downward *et al.*, 2011). Lung bacteria originate from the upper respiratory tract and the mouth (Bassis *et al.*, 2015; Venkataraman *et al.*, 2015). The question of a stable versus transient colonization of lung by bacteria remains open (Segal *et al.*, 2013), but even if the lung microbes are only transited they

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can have major health impact. The composition and role of the lung microbiota remain largely unknown, and has mainly been explored using genomic approaches (Beck *et al.*, 2012; Charlson *et al.*, 2012). In 2011, Sibley *et al.* evaluated the cultivability of the airway microbiome using culture-enriched molecular profiling and were able to cultivate 43 of the 48 families detected by deep sequencing. Using both culture and genomic approaches, Yun *et al.* (2014) have shown that the lung microbiota is diversified through different environmental conditions and affects lung architecture. The direct contribution of the lung microbiota, and potential associated dysbiosis, in both the physiology and pathology of the respiratory tract remains unclear.

Recent publications suggest that the lung microbiota is altered in asthmatic patients. Analysis of 16S RNA sequences in bronchial lavage from asthmatic children revealed a highly significant increase of *Proteobacteria* (Hilty *et al.*, 2010). Moreover, airway dysbiosis appears to vary depending on the severity of the disease (Huang *et al.*, 2015) or corticosteroid responsiveness (Goleva *et al.*, 2013). The correlation between asthma and modification of the airway microbiota composition is now clearly established (Teo *et al.*, 2015; Smits *et al.*, 2016). Gollwitzer *et al.* (2014) demonstrated that microbial signals in the lung of neonatal mice clearly improve immune tolerance to House Dust Mite (HDM) allergens via PD-1/PD-L1 signaling in regulatory T cells and dendritic cells (DC). Their study established a direct contribution of microbial components to HDM induced asthma.

Previous studies proposed that asthma features are influenced by microbiota based on ovalbumin challenge model (Herbst *et al.*, 2011; Olszak *et al.*, 2012) or oral antibiotic treatment (Hill *et al.*, 2012). The contribution of lung bacterial strains to the establishment of asthma or their participation in chronic inflammation has never been addressed by using HDM challenge. In this study, we demonstrate that lung bacteria participate in determining the immune and inflammatory responses to HDM in neonatal C57BL/6 mice.

## Materials and methods

### *Bacterial strains, media, growth conditions*

Lung bacterial strains were isolated from mouse lung homogenates prepared using an Ultraturax or Tissue Lyser (Qiagen, Courtaboeuf, France), diluted in Brain Heart Infusion liquid medium supplemented with 5 g l<sup>-1</sup> of yeast extract, 5 mg l<sup>-1</sup> of hemin, 2 mg l<sup>-1</sup> of vitamin K1 and 0.5 g l<sup>-1</sup> of cysteine (yhBHI, all products are from Sigma-Aldrich, Eurogentec, Angers, France) and cultivated on agar yhBHI medium (GyhBHI), M17, MRS or Mannitol Sel Agar medium for 24–48 h at 37 °C under aerobic conditions or 5 days at 37 °C in a Freter chamber under

anaerobic conditions. Isolated strains were frozen at -80 °C in 16% glycerol. Strain identities were confirmed by mass spectrometry, API gallery (BioMérieux, Craponne, France) and 16S PCR sequencing. Selected strains were deposited at the French National Collection of Microorganism Cultures (CNCM) under the name CNCM I 4969 and CNCM I 4970 (patented strains).

### *Animals and procedures*

Animal experiments were approved by the local ethics committee under the registration number 01553.01. SPF C57BL/6 mice purchased from Janvier (Le Genest, St Isle, France) were bred and housed under FELASA SPF conditions in our animal facilities (IERP, INRA, Jouy-en-Josas, France, or VIB, Ghent, Belgium). GF C57BL/6 mice were purchased from CDTA (CNRS, Orleans, France) or local breeders (INRA) and were bred and housed under germ-free conditions in Trexler-type isolators (La Calhène, Vélizy, France) in the Anaxem animal facilities (INRA). For HDM allergic asthma induction, 7-day-old pups received 1 µg of HDM (Greer, Lenoir, NC, USA) or diluent lipopolysaccharide-free phosphate-buffered saline (PBS) (Lonza, Levallois-Perret, France) in a volume of 10 µl. They were challenged 1 week later with 10 µg of HDM (or PBS) for five consecutive days. In some experiments, 5-day-old pups received 1 × 10<sup>6</sup> bacteria in 10 µl PBS every 2 days.

### *Sample collection*

Mice were killed by intraperitoneal injection of a lethal dose of ketamine and xylazine. The left bronchus was clamped and bronchoalveolar lavage (BAL) was performed on the right lobes with PBS 1 mM EDTA as described (Roux *et al.*, 2011). BAL supernatants were stored frozen at -20 °C and BAL cells were cytocentrifuged (Cytospin 5) on microscope slides (Superfrost, Thermo Scientific, Braunschweig, Germany), and then stained with May-Grünwald and Giemsa. The right lobe was used for flow cytometry or bacterial enumeration on agar plates after homogenization with a Tissue Lyser. For flow cytometry, the right lobe and the respiratory lymph nodes (RLN) (cervical, maxillary and mediastinal LN) were treated with 1 mg ml<sup>-1</sup> collagenase D and 0.5 mg ml<sup>-1</sup> DNase I for cell isolation. The left lobe of the lung was kept frozen at -80 °C until processing for RNA extraction. For histological examination, the lungs were fixed with 4% paraformaldehyde and embedded in paraffin.

### *Precision-cut lung slides*

Precision-cut lung slides (PCLS) were obtained from fresh lungs using a Krumdieck tissue slicer MD 6000 (Alabama Research and Development, Munford, AL, USA). The lungs were filled via the trachea with



RPMI 1.5% low melting point agarose (Invitrogen, Villebon sur Yvette, France) warmed to 37 °C. After 1 min for solidification, the lungs were placed in the microtome chamber of the Krumdieck, filled with cold PBS and cut at a thickness of 200 µm. Two PCLS per well were then placed at 37 °C, 5% CO<sub>2</sub>, in P24 well plates (Nunc, Sigma-Aldrich, Lyon, France) with 1 ml RPMI 1640 (Gibco, Sigma-Aldrich, Lyon, France) supplemented with 10% heat inactivated fetal calf serum (Gibco) and 2 mM L-glutamine (Gibco). The medium was changed every 30 min during 2 h to remove the low melting point agarose and one last time after overnight incubation. PCLS were then co-incubated for 24 h with lung bacteria. Viability was assessed by measuring lactate dehydrogenase release (Sigma) or by MitoTracker Deep Red (ThermoFisher, Illkirch, France) labeling.

#### Histology

Five-micrometer lung sections were stained with hematoxylin/eosin/saffron or periodic acid schiff/alcian blue and photographed using CaseViewer software (3DHISTECH, Budapest, Hungary).

#### Gene expression by quantitative reverse transcriptase PCR

Total RNA was extracted from mouse lung homogenates using the NucleoSpin RNA kit (Macherey Nagel, Düren, Germany) and reverse transcribed using random primers and 50 U of Reverse Transcriptase (High-Capacity cDNA Archive Kit; Applied Biosystems by Life Technologies SAS, Villebon Sur Yvette, France) according to the manufacturer's instructions. The primers (Sigma-Aldrich) are listed in Supplementary Table S1. Quantitative reverse transcriptase PCR was performed in triplicate for each gene using an AbiPrism 7000 (Applied Biosystem) and Takyon ROx SYBR MasterMix (Eurogentec) and the data were analyzed using 700 System SDS software (Applied Biosystem) to determine the cycle threshold (Ct) values. Messenger RNA (mRNA) expression was calculated using the ΔCt method and normalized to the expression of mHPRT.

#### Flow cytometry

After saturation with anti-CD32/CD16, cells were incubated with mAbs reactive to mPDCA1 (JF05-1C2.4.1, conjugated to FITC), MHCII (IA/IE, 2G9 or M5/114.15.2, FITC or PE), CD103 (M290 or 145-2D11, PE), CD86 (GL1, FITC), CD11b (M1/70, PerCP Cy5.5) or CD11c (HL3, Biotin or BV786). For T-cell staining, cells were incubated with mAbs reactive to CD4 (L3T4 or RM4-5, FITC or PECy7), CD45.2 (104, A780), CD3 (145-2C11, PerCP Cy5.5) or CD8 (Ly2, 53-6.8, Biotin or APC). For B-cell staining, cells were incubated with mAbs reactive to CD19 (1D3, APC), B220 (RA36B2, APC), CD45.2 (104, A780), CD5

(53-7.3, BV421) or CD23 (B3B4, FITC). All mAbs were purchased from BD Biosciences (San Jose, CA, USA) except for mPDCA1 (Miltenyi Biotec, Paris, France). APC-conjugated streptavidin (BD Biosciences) was used to label biotin Abs. At least 2 × 10<sup>6</sup> events were acquired using an Accuri or Fortessa FACS (BD Biosciences) and analyzed using FlowJo Software v7.5 (Tree Star Inc., Ashland, OR, USA).

#### Cytokine and Ig enzyme-linked immunosorbent assay

The cytokines IL-5, IL-10, IL-12p70, IL-17a or IFNγ (Mabtech, Nacka Strand, Sweden) and TSLP (eBiosciences, Paris, France) were quantified by enzyme-linked immunosorbent assay according to the manufacturer's instructions. Individual mouse sera were assayed for IgE and IgG1 (eBiosciences) according to the manufacturer's instructions.

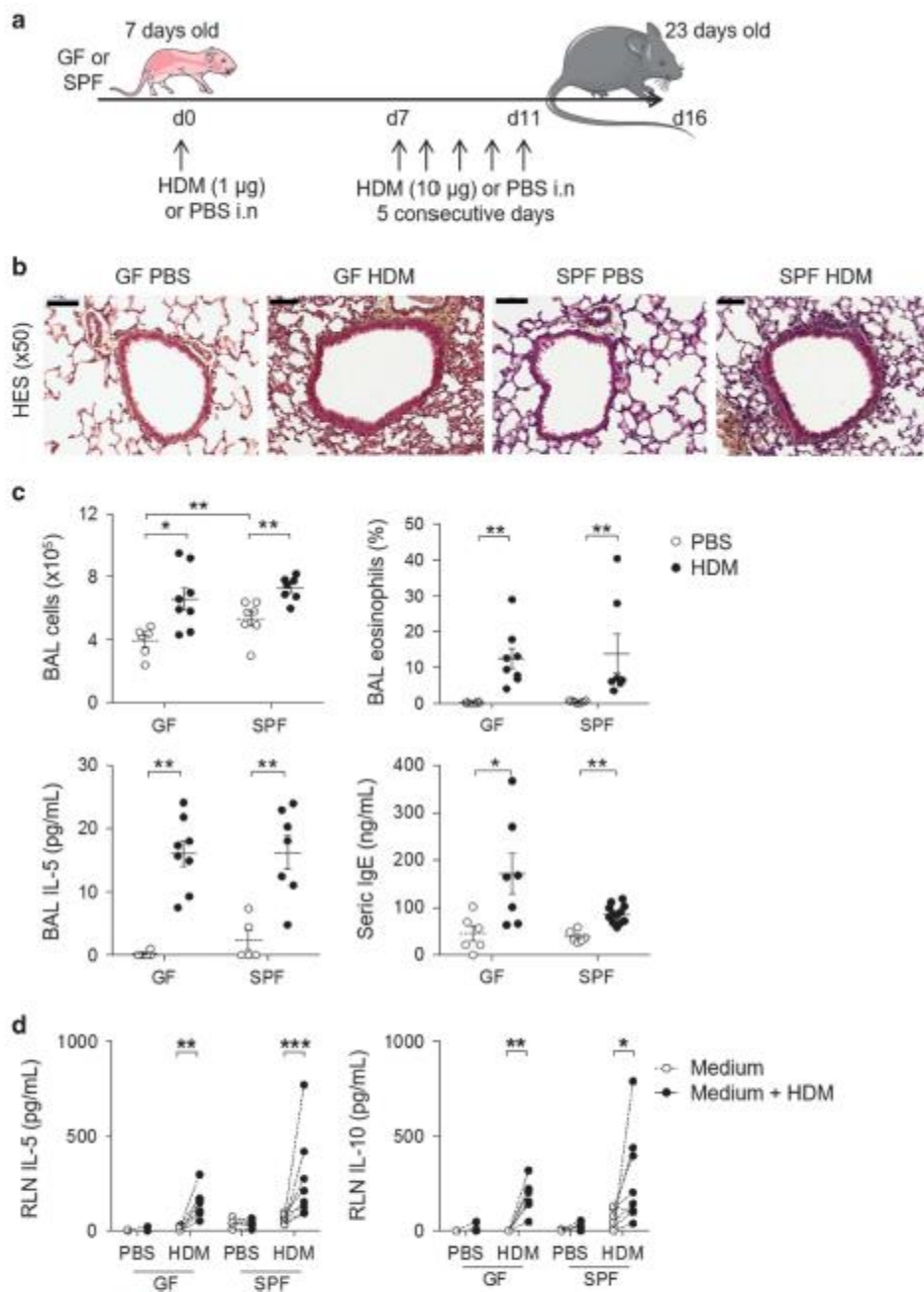
#### Statistical analysis

Non-parametric Mann-Whitney (comparison of two groups,  $n \geq 4$ ) or ANOVA Tukeys multiple comparison test ( $> 2$  groups) was used to compare unpaired values (GraphPadPrism software, GraphPad Software, Inc., San Diego, CA, USA). Significance is represented: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; and \*\*\*\* $P < 0.0001$ .

## Results

#### Allergic asthma is not modified in germ-free mice

Asthma sensitivity was evaluated both in Germ-Free (GF) and Specific-Pathogen-Free (SPF) neonatal mice (Figure 1a). On day 0, 7-day-old neonates were intranasally sensitized with 1 µg of HDM or PBS diluent (PBS). They were challenged 1 week later with 10 µg of HDM or PBS intranasally on five consecutive days. All mice were killed, when they were 23 days old (5 days after the last challenge dose) to assess the inflammatory and immune responses in young adults (Figures 1b–d). *In vivo* lung function measurements were not feasible at that age. Both GF and SPF HDM challenged mice showed similar levels of inflammatory cell recruitment near the airway, bronchus epithelium thickening and myofibroblasts by hematoxylin eosin safran coloration of lung sections (Figure 1b). We did not observe any striking differences in lung structure, airway epithelium thickness or bronchus number between GF PBS- and SPF PBS-treated mice (Figure 1b), although the lungs of GF mice seemed to have fewer, larger alveoli, as previously published (Yun *et al.*, 2014). The total number of cells recovered from BAL was greater after HDM treatment (Figure 1c, PBS vs HDM:  $P < 0.05$  for GF;  $P < 0.01$  for SPF). We observed a type 2 immune (T2) signature after HDM treatment, as shown by the presence of eosinophils and IL-5 in BAL (Figure 1c, PBS vs HDM:  $P < 0.01$  for both GF and SPF), IgE in sera (Figure 1c, PBS vs HDM:  $P < 0.05$  for GF,  $P < 0.01$  for SPF), and IL-5 and IL-10



**Figure 1** HDM-induced asthma is not exacerbated in GF mice. (a) Schematic outline of the experimental approach. Animals were killed on d16. (b) Lungs were fixed, embedded in paraffin and sectioned at a thickness of 5 µm. Lung sections were stained with hematoxylin-eosin-saffron (HES) and photographed using Case Viewer software. One representative section per group is shown ( $n=6$  GF mice,  $n=11$  SPF mice). The scale bar represents 50 µm. (c) BAL cells were enumerated, cytocentrifuged and stained with May-Gründwald-Giemsa. Eosinophils were enumerated and expressed as the percentage of total BAL cells. BAL IL-5 and serum IgE levels were measured by enzyme-linked immunosorbent assay (ELISA). Data are shown individually and as the mean  $\pm$  s.e.m. (d) Individual respiratory lymph node (RLN) cells were isolated and cultivated 72 h in RPMI with or without HDM. IL-5 and IL-10 levels in the supernatants were measured by ELISA. The RPMI  $\pm$  HDM values for the same mice are connected with a dotted line. Data are shown individually and as the mean  $\pm$  s.e.m. (a-d) All data represent one of two independent experiments ( $n=4-8$  mice per group).

secretion by RLN cells re-stimulated *in vitro* with RPMI medium supplemented with HDM (Figure 1d,  $P<0.01$  (both) for GF;  $P<0.001$  (IL-5) and  $P<0.05$  (IL-10) for SPF). HDM efficiently induced allergic asthma, with an equivalent magnitude of T2

immunity and inflammatory response in GF and SPF mice, despite higher variability of IgE levels in GF sera.

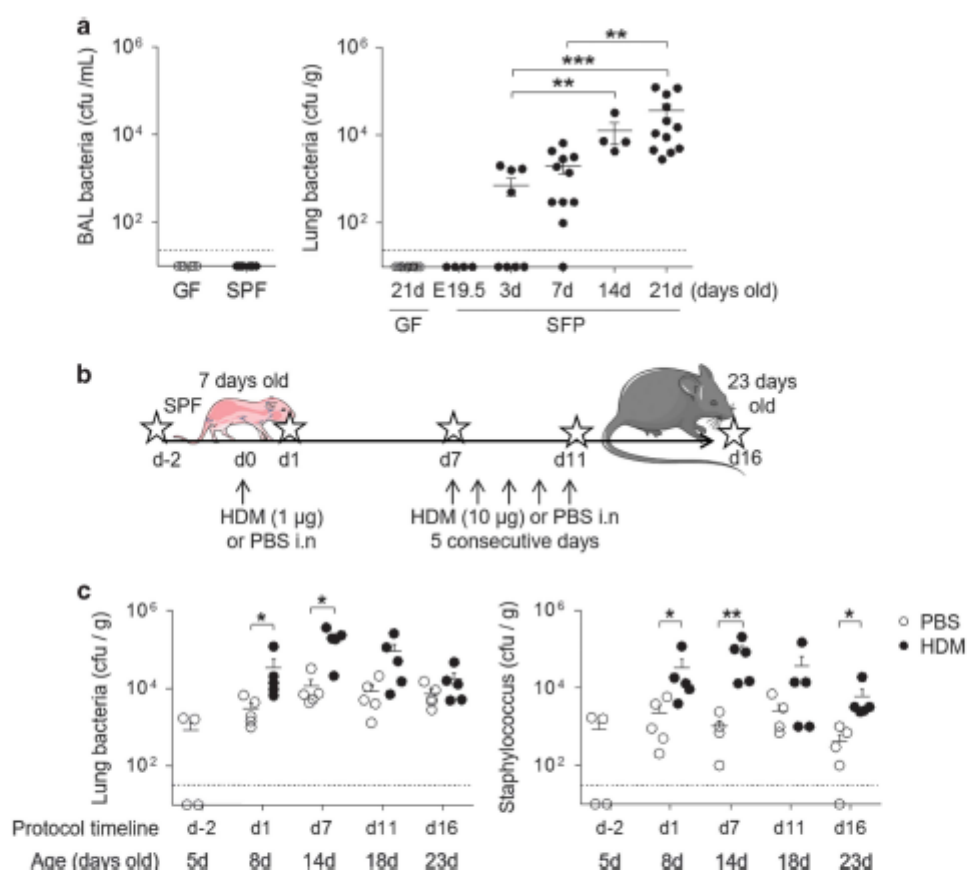
We observed that some innate genes showed different patterns of expression at steady state

between GF and SPF mice: the level of *TSLP* mRNA was higher in GF mice, whereas *IL-10*, *IFN $\gamma$* , *CCL11* and *Muc5AC* mRNA levels were higher in SPF mice (Supplementary Figure S1,  $P < 0.05$  for *IL-10*,  $P < 0.01$  for all other genes). We next evaluated the number of lung immune cells, such as DC, T and B cells, by flow cytometry (Supplementary Figures S2A–D). All subpopulations of DCs, including plasmacytoid DCs (pDC) and the two subsets of conventional DCs (cDC) CD11b<sup>+</sup> CD103<sup>-</sup> (CD11b<sup>+</sup>) and CD11b<sup>-</sup> CD103<sup>+</sup> (CD103<sup>+</sup>), were present at the same levels in both GF and SPF lungs. The levels were also similar for CD4 and CD8 T cells, and B cells.

Altogether, our results show differences in mRNA levels for some innate genes between GF and SPF mice, but no marked differences of lung physiology or immune cell numbers (except for higher BAL cellularity for SPF mice, Figure 1c,  $P < 0.01$ ). HDM-induced allergic asthma was neither reduced nor exacerbated in GF mice. The absence of bacteria had no obvious influence on HDM pathology.

### Asthma influences lung microbiota during the neonatal period

We tested whether HDM-induced asthma influences the viable bacteria amount in the lung. We used samples from embryonic day 19.5 and GF mice as sterile controls for our culture protocol. After birth, bacteria gradually arrived to the lung (Figure 2a). The bacteria can be cultivated both in aerobic and anaerobic conditions (data not shown). We never obtained cultivable bacteria from individual BAL of SPF neonatal mice (Figure 2a) nor purified enough bacterial DNA for 16S sequencing (data not shown). We succeeded in recovering viable bacteria from the lungs of SPF mice starting from 3 days after birth, and the number of lung bacteria increased significantly each week until weaning after 21 days (Figure 2a;  $P < 0.01$  and  $P < 0.001$ ). We isolated more than 20 bacterial strains from neonatal lungs: a majority of members within the Firmicutes phylum (*Staphylococcus*, *Streptococcus*, *Enterococcus*, *Listeria*, *Lactobacillus*) and some from the Proteobacteria phylum (*Escherichia coli*, *Proteus mirabilis*) (Supplementary Table S2).



**Figure 2** Lung microbiota is modified during HDM induced asthma. (a) BAL or lung homogenates were incubated 24 h on GyhBHI plates and the bacteria enumerated as colony-forming units (CFU per ml of BAL (from 21-day-old GF or SPF mice) or grams of lung (from embryonic day 19.5 embryos or 3, 7, 14 or 21-day-old neonates)). (b) Schematic outline of the experimental approach for panel (c) (days of killing are illustrated with stars). (c) Lung homogenates were incubated 24 h on GyhBHI (to enumerate all bacteria CFU) and Mannitol Sel agar plates (to enumerate *Staphylococcus* CFU). (a–c) All data represent one of three independent experiments ( $n = 5–10$  mice per group).



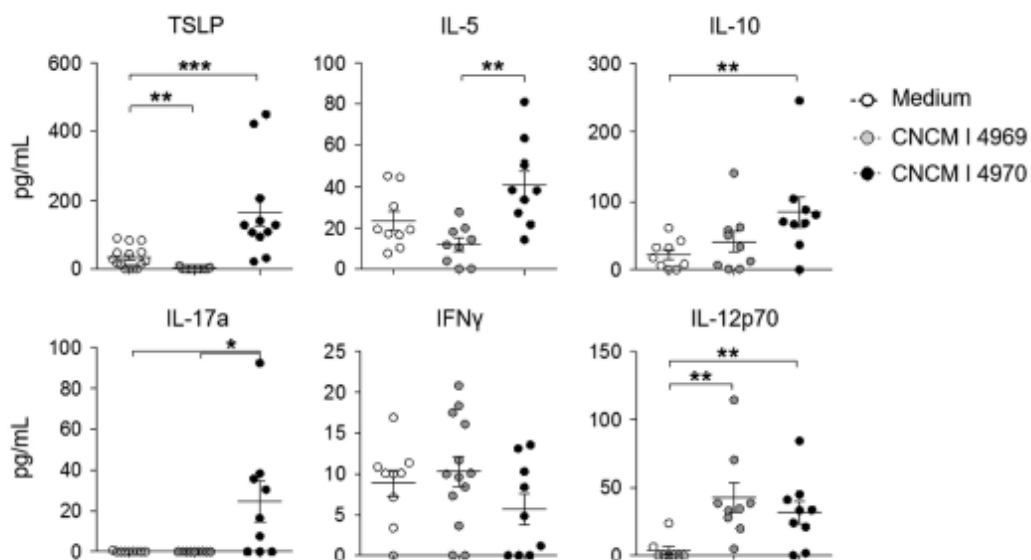
To evaluate the impact of HDM treatment on the lung microbiota, mice were killed at different time points as illustrated by stars on the time line in Figure 2b. The number of bacteria recovered in non-selective GyhBHI medium significantly increased on d1 and d7 (Figure 2c,  $P < 0.05$ ). This tendency was not significant on d11, and the number of colony-forming units (CFU) reached similar levels for HDM and SPF mice on d16. *Staphylococci* levels were particularly high after HDM treatment (Figure 2c, selective Mannitol Sel Agar plates). Thus, HDM treatment influenced the composition of the lung microbiota during the neonatal period.

#### Lung bacteria influence asthma features

We next sought to screen the 20 lung bacterial strains for their capacity to differentially promote cytokine production by PCLS. Of the 20 strains tested, 7 induced significant production of cytokine (Supplementary Table S2) and CNCM I 4969 and CNCM I 4970 (patented strains, Gram<sup>+</sup> coccus) displayed specific immunostimulatory properties (Figure 3). After 24 h of co-culture with GF PCLS, CNCM I 4969 stimulated the secretion of IL-12p70 and decreased the basal level of TSLP (Figure 3,  $P < 0.01$ ). CNCM I 4970 significantly increased the amount of TSLP, IL-10, IL-17a and IL-12p70 released into PCLS supernatants (Figure 3,  $P < 0.001$  for TSLP,  $P < 0.01$  for IL-10 and IL-12p70, and  $P < 0.05$  for IL-17a). The secretion of IL-5 tends to be higher in CNCM I 4970-stimulated cells compared with non-stimulated cells, but does not reach significant level ( $P = 0.0625$ ). Altogether, CNCM I 4969 induced a

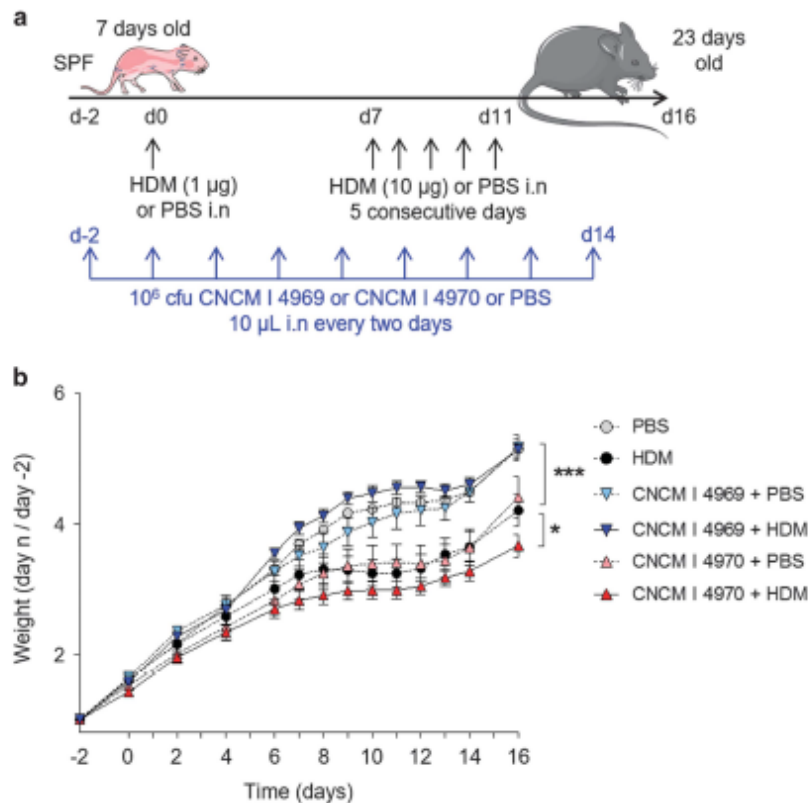
type 1 cytokine profile whereas CNCM I 4970 (a member of the *Staphylococcus* genus, whose levels were higher after HDM treatment) induces secretion of the majority of cytokines tested (TSLP, IL-10, IL-17a and IL-12p70).

We next investigated whether these two immunomodulatory bacteria isolated from the neonatal lung microbiota could affect the severity of HDM-triggered asthma in SPF mice. Neonates received  $10^8$  CFU of CNCM I 4969 or CNCM I 4970 (10  $\mu$ l intranasally every 2 days starting at d2), and the readouts of asthma features were explored after weaning in young adult mice (23 days old) as illustrated in Figure 4a. Nasal administration enabled bacterial delivery to the lungs, as verified by the detection of CFDA-SE labeled strains in BAL and lungs (Supplementary Figure S3). HDM treatment induced a small, but reproducible ( $n = 3$  experiments), growth delay in neonates (Figure 4b, PBS vs HDM,  $P < 0.001$ ). Indeed, during the challenge phase (d7 to d11), HDM-treated neonates gained less weight. CNCM I 4969 administered alone had no significant effect on the growth of the pups. CNCM I 4970 alone negatively affected weight gain (Figure 4b, PBS vs CNCM I 4970). This result was obtained for two of three experiments (data not shown). In the HDM groups, CNCM I 4969 protected the mice against the growth delay (Figure 4b,  $P < 0.001$ ) whereas CNCM I 4970 worsened it (Figure 4b,  $P < 0.05$ ). We observed fewer infiltrating cells in BAL from the CNCM I 4969+HDM group (Figure 5a, CNCM I 4969+HDM group vs HDM,  $P < 0.001$ ), and the percentage of eosinophils was lower (Figure 5a,  $P < 0.05$ ). The challenge with



**Figure 3** Ex vivo immunomodulation profiles of CNCM I 4969 and CNCM I 4970. Precision-cut lung slices (PCLS) of adult GF lungs were cultivated *ex vivo* for 24 h in supplemented RPMI alone (medium) or with 50 CFU of the two strains, CNCM I 4969 and CNCM I 4970, isolated from healthy neonatal lungs. Cytokines were measured in PCLS supernatants. Data represent two pooled independent experiments ( $n = 9$  mice per group). In total, four independent experiments were performed with similar results ( $n = 4-5$  mice per group per experiment).





**Figure 4** Bacterial intervention can modulate asthma features in SPF mice. **(a)** Schematic outline of the experimental approach. Animals were killed on d16. **(b)** Mice were weighed daily. The growth curves are expressed as the mean  $\pm$  s.e.m. of individual weights (normalized to the initial weight on day -2) for  $n \geq 5$  mice per group and represent one of three independent experiments ( $n = 5-10$  mice per group). Tukey's multiple comparison test, repeated measures one-way ANOVA was used for comparison of the growth curves.

HDM increased the infiltration of neutrophils and lymphocytes (Figure 5a,  $P < 0.01$ ) that remained unchanged in the presence of our strains. The strains CNCM I 4970 significantly increased the basal level of neutrophils and lymphocytes (Figure 5a, PBS vs CNCM I 4970,  $P < 0.05$  and  $P < 0.01$ ). In our model, the response is due to eosinophil recruitment rather than neutrophil. CNCM I 4970 increased IL-5 levels in PBS-treated mice (Figure 5a,  $P < 0.05$ ) but they were similar in the HDM and CNCM I 4970+HDM groups. The basal level of IgE was increased by both strains, in the absence of HDM (Figure 5b, PBS vs CNCM I 4970 and PBS vs CNCM I 4969,  $P < 0.01$ ). The treatment with HDM led to a higher amount of serum IgE (Figure 5b, HDM vs PBS,  $P < 0.05$ ) and this HDM-induced IgE was worsened by CNCM I 4970 +HDM (Figure 5b, HDM vs CNCM I 4970+HDM,  $P < 0.01$ ). The serum IgE levels were not different in HDM and in CNCM I 4969+HDM, thus indicating that CNCM I 4969 has no impact on HDM-induced IgE. IgG1 levels were increased in the presence of HDM plus each strains (Figure 5b, HDM vs CNCM I 4970+HDM,  $P < 0.01$ ; HDM vs CNCM I 4969+HDM,  $P < 0.05$ ). In RLN supernatants, IL-5 and IL-10 production was significantly lower in the CNCM I 4969+HDM group than in the HDM group (Figure 5c,

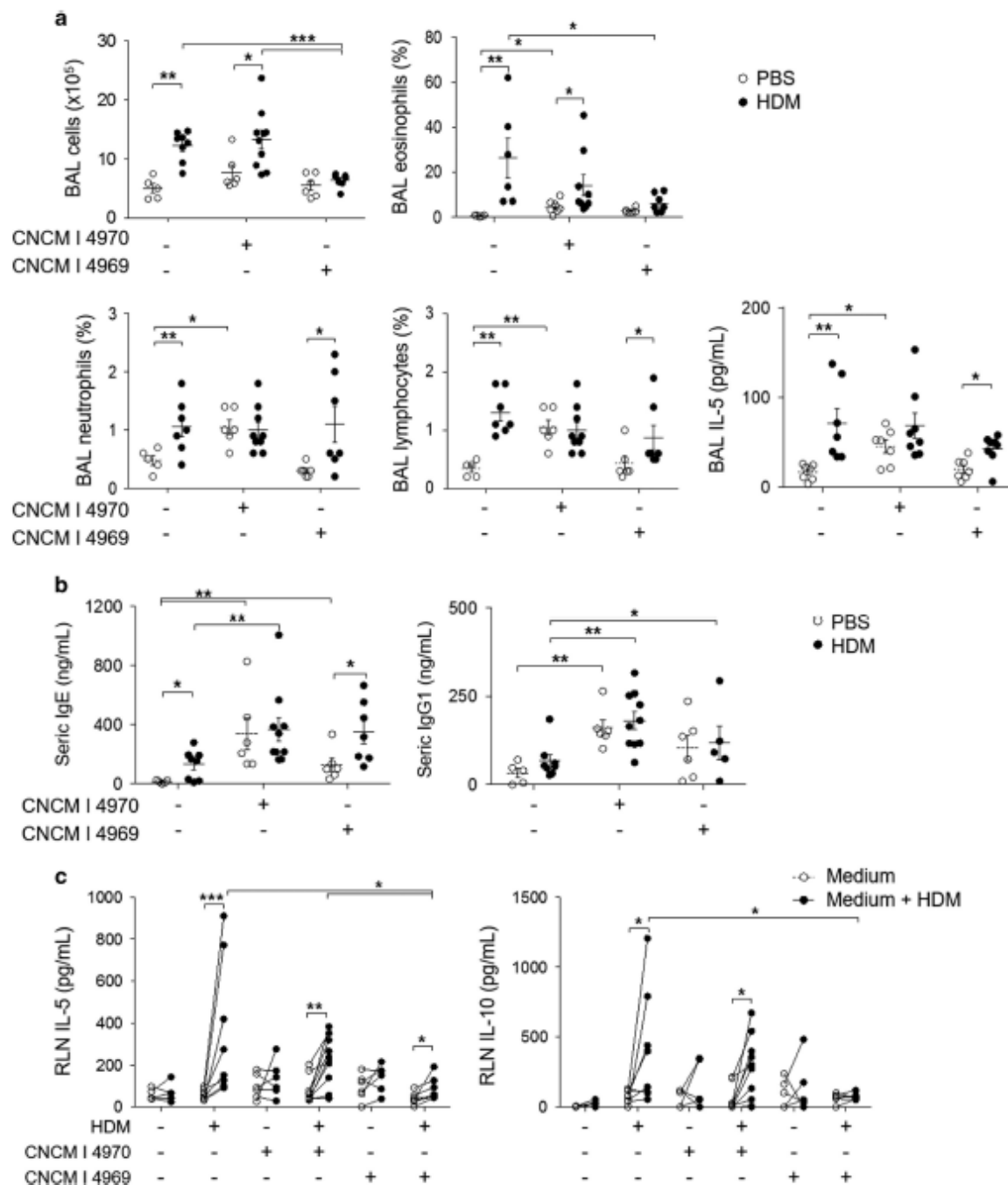
$P < 0.05$ ), suggesting reduced type 2 immune priming.

The lungs from HDM-treated mice showed perivascular immune infiltrates, myofibroblast proliferation (Figure 6a, hematoxylin eosin safran staining), mucus production (Figure 6a, periodic acid schiff/alcian blue staining) and a thickened epithelium relative to PBS-treated mice (Figure 6b,  $P < 0.0001$ ). A similar pattern of inflammatory lesions was observed for the CNCM I 4970 group, which increased moderately upon HDM challenge. In contrast, CNCM I 4969 did not cause lung inflammation by itself and even reduced the extent of the inflammatory reaction in the tissue upon HDM challenge. All features were markedly reduced relative to the HDM or CNCM I 4970+HDM groups (Figures 6a and b,  $P < 0.0001$ ). Thus, inoculation with CNCM I 4969 protected mice from HDM-induced airway disease.

We obtained an intermediate profile when we co-administered CNCM I 4969 and CNCM I 4970. The mice were not protected against the growth delay (Figure 7a, HDM vs CNCM I 4969+4970+HDM = NS). Serum IgE and BAL IL-5 levels were similar (Figure 7b), but BAL cellularity was reduced ( $10.39 \pm 0.45 \times 10^5$  in HDM vs  $7.32 \pm 0.75 \times 10^5$  in CNCM I 4969+4970+HDM,  $P < 0.01$ ) as well as the percentage of BAL eosinophils (Figure 7b,  $P < 0.05$ ).

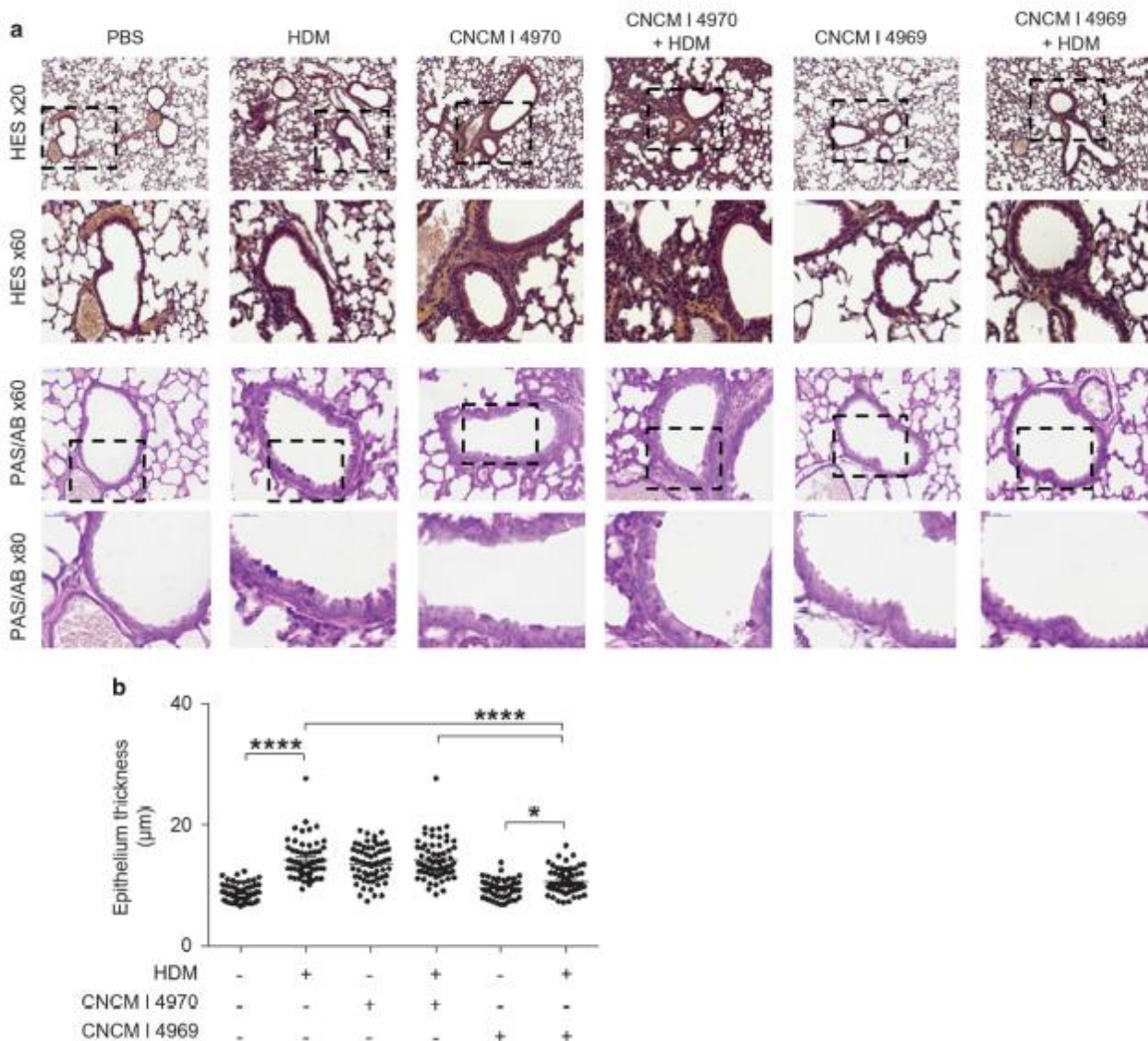
IL-5 levels were reduced in RLN supernatants after *in vitro* HDM re-stimulation (Figure 7c,  $P < 0.01$ ), reflecting reduced type 2 immune priming. Finally, histological examination of lung tissue revealed moderate inflammation, with a profile comparable to

the HDM group (Figure 7d vs Figure 6). CNCM I 4969 appeared to attenuate some enhanced asthma features induced by CNCM I 4970 alone. Note that the strain CNCM I 4967 (*Lactobacillus* spp), also isolated from mouse lung, had neutral effect on HDM-



**Figure 5** CNCM I 4969 decreases some type 2 immune features whereas CNCM I 4970 worsens it. SPF mice were treated as described in Figure 4. (a) BAL cells were enumerated, cytocentrifuged and stained with May-Gründwald-Giemsa. Eosinophils, neutrophils and lymphocytes were enumerated and expressed as the percentage of total BAL cells. BAL IL-5 levels were measured by enzyme-linked immunosorbent assay (ELISA). (b) Serum IgE and IgG1 levels were measured by ELISA. (a, b) Data are shown individually and as the mean  $\pm$  s.e.m. (c) Individual respiratory lymph node (RLN) cells were isolated and cultivated 72 h in RPMI with or without HDM. IL-5 and IL-10 levels in the supernatants were measured by ELISA. The RPMI  $\pm$  HDM values for the same mice are connected with a dotted line. (a-c) All data represent one of three independent experiments ( $n = 5-10$  mice per group).





**Figure 6** CNCM I 4969 protects against lung inflammation whereas CNCM I 4970 worsens it. SPF mice were treated as described in Figure 4. (a) Lung histology was assessed as described in Figure 1. Lung sections were stained with hematoxylin–eosin–safran (HES, first two rows, enlargement  $\times 20$  and  $\times 60$ ) or periodic acid–Schiff and alcian blue (PAS/AB, bottom two rows, enlargement  $\times 60$  and  $\times 80$ ). One representative section per group is shown. (b) Epithelium thickness was measured using Case Viewer software. In total, 100 measures per group are plotted on the graph. They correspond to the analysis of four mice per group. Five representative bronchi were selected for each mouse and four measurements were performed per bronchus (up, down, left, right side). (a, b) All data represent one of two independent experiments ( $n = 5–10$  mice per group).

induced asthma (Supplementary Table S2 and data not shown).

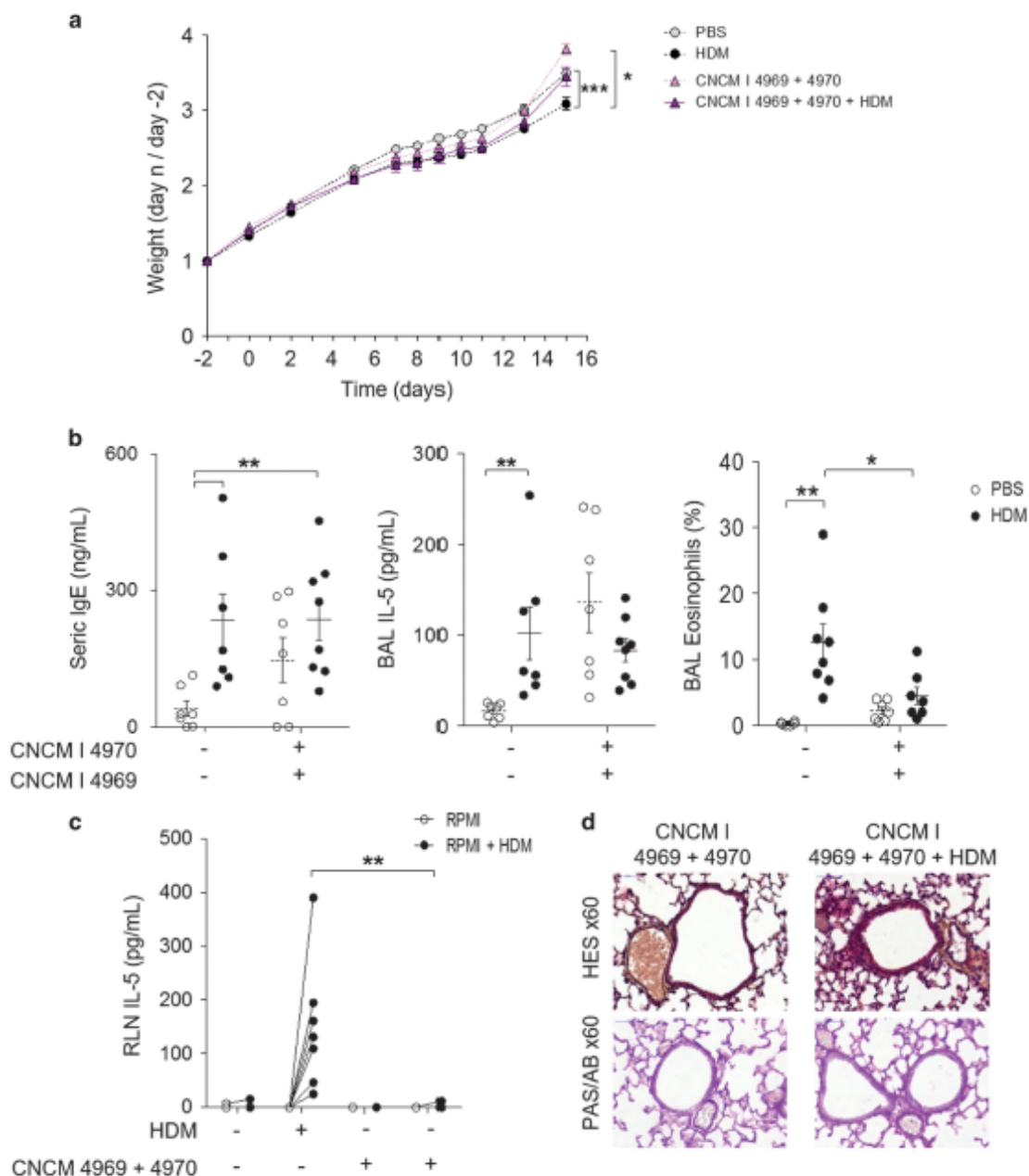
Altogether, our results show that forced inoculation of lung bacteria influences the severity of HDM-induced allergic asthma: CNCM I 4970 exacerbated some asthma features whereas CNCM I 4969 seemed to have a protective effect.

## Discussion

Our study provides evidence for a reciprocal influence between lung bacteria and asthma in neonatal mice. Our data underline the bacterial hypothesis in asthma

(Figure 8) as the early lung bacteria environment can worsen or attenuate asthma features.

We provide evidence for the presence of living bacteria in healthy mouse lungs. The number of viable bacteria increased slightly, but significantly, within the first 2 weeks. To our knowledge, only one other study has described the culture of living bacterial strains from adult mouse lungs (Yun *et al.*, 2014). In agreement with this study, we also found members of Enterobacteria and Firmicutes. Lung microbiota has been mostly investigated using genomic tools. Here, we used bacterial culture to further study the functional and immunomodulatory properties of lung bacteria.

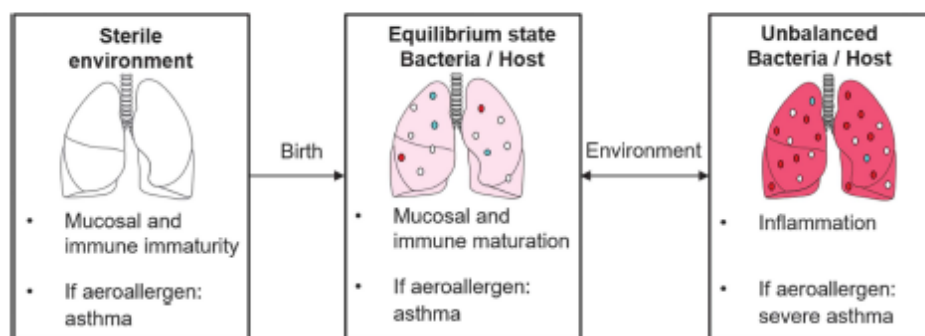


**Figure 7** CNCM I 4969 and 4970 induce an intermediate inflammatory profile when given together. SPF mice were treated as described in Figure 4 but the two strains were inoculated together. (a) Mice were weighed daily. The growth curves are expressed as the mean  $\pm$  s.e.m. of individual weights (normalized to initial weight at day -2). (b) Serum and BAL responses were analyzed as described in Figures 5a and b. Data are shown individually and as the mean  $\pm$  s.e.m. (c) IL-5 was measured in RLN supernatants as described in Figure 5c. The RPMI  $\pm$  HDM values for the same mice are connected with a dotted line. (d) Lung histology was assessed as described in Figure 1. Lung sections were stained with hematoxylin–eosin–safran (HES) or Periodic acid–Schiff and alcian blue (PAS/AB). One representative section (enlargement  $\times 60$ ) per group is shown. (a–d) All data represent one experiment with  $n = 7–8$  mice per group.

The presence of the microbiota obviously influences local lung physiology and immunity. We found that innate mRNA markers were differentially expressed at steady state between the lungs of GF and SPF mice and that BAL cellularity was higher in SPF mice. Histological examination of lung slices showed no major defect in the lungs of GF mice. On the contrary to what we observed in lung, the gut

microbiota triggers extensive remodeling of the epithelium of the small and large intestines (El Aidy *et al.*, 2012; Tomas *et al.*, 2013; Hoffmann *et al.*, 2016). One important difference between gut and lung epithelia is their renewal, which is very active in the gut (Sancho *et al.*, 2004). The presence of stem and proliferative daughter cells allows constant reshaping of the gut to face a highly variable luminal





**Figure 8** Lung microbiota is an early life determinant of susceptibility to allergic asthma. Lung bacteria environment affects lung physiology and immunity, and the equilibrium between the host and its microbiota influences microbiota composition and asthma features.

environment (Peifer, 2002; Joly *et al.*, 2009; Kaiko and Stappenbeck, 2014). For example, gut epithelial cell turnover is an efficient and effective mechanism for pathogen expulsion (Cliffe *et al.*, 2005). The low-level renewal of lung cells may be linked to the low influence of the microbiota on its structure.

The number of lung DC recovered from the lungs of GF and SPF mice in our experiments was similar, in agreement with Gollwitzer *et al.* (2014). These authors have also observed higher expression of CD40 and PD-L1 DC markers in 8-day-old SPF mice, showing a more mature phenotype in the presence of microbes. We have also found similar numbers of lung B and T lymphocytes, but did not assess the different helper T-cell subsets that have been shown to be regulated by the microbiota (Gollwitzer *et al.*, 2014; Ohnmacht *et al.*, 2015). Gollwitzer *et al.* (2014) reported no difference in lung DC and CD4 T cells frequency in GF and SPF mice following HDM exposure. In our hands, the same conclusion remained true (data not shown, one experiment).

GF mice have altered T- and B-cell numbers in the gut (Macpherson and Harris, 2004; Hooper and Macpherson, 2010). This is not the case in the lung. We hypothesize that lung regulatory mechanisms are less strongly affected by the microbiota, possibly due to a lower bacterial load:  $10^{12}$  per gram in the colon (Costello *et al.*, 2009; Marchesi, 2011) vs  $10^4$  per gram in the lung (Figure 3a). Some bacterial families specifically affect gut immunity and physiology (Wrzosek *et al.*, 2013; Miquel *et al.*, 2015b; Tomas *et al.*, 2015). For example, segmented filamentous bacteria increase Th17 (Lécuyer *et al.*, 2014). It is possible that family-specific regulation also occurs in the lung, as we observed different immune-regulating properties *ex vivo* of different bacterial strains.

Bacteria can be ranked into functional cores according to their function (Lozupone *et al.*, 2012) and it is well described that beneficial or detrimental effects of bacteria are specific to the strains considered (Miquel *et al.*, 2015a; Peres *et al.*, 2015). It may be instructive to link the effects of lung bacteria to their metabolic activity (Miquel *et al.*, 2015b).

Indeed, we observed *in vitro* short-chain fatty acid production by the 20 strains that we studied: mainly acetate ( $5\text{--}60\text{ mmol l}^{-1}$ ), and low levels of propionate and butyrate ( $< 5\text{ mmol l}^{-1}$ ) for some strains (data not shown). CNCM I 4969 and CNCM I 4970 produce the same amount of acetate ( $5\text{--}6\text{ mmol l}^{-1}$ ) and no propionate or butyrate *in vitro* so it is unlikely that the difference observed on asthma pathology is due to a difference in short-chain fatty acid production.

The lung microbiota enters the airway within a few days and others have suggested that its composition stabilizes after 2–3 months in infants (Marsland, 2013). During this primocolonization process, the neonatal period represents a window of susceptibility to infections by pathogens, or the development of non-infectious diseases such as allergic asthma. Early and chronic exposure to microbial products such as endotoxin has been demonstrated to prevent allergic asthma in children growing up in a dairy farm (Schuijs *et al.*, 2015). Endotoxins reduce the production of DC-activating cytokines by epithelial cells, thus suppressing type 2 immunity to HDM (Schuijs *et al.*, 2015). Here, early administration of either pro-type 1 or pro-type 2 strains modulated aeroallergen responsiveness. Although we were able to reduce the type 2 immune response with CNCM I 4969, we did not observe increased production of type 1 cytokines IL-12p70 or IFN $\gamma$  in RLN supernatants or BAL fluids (data not shown). TSLP levels in neonatal BAL fluids were under our detection limit. We also observed the presence of IL-17a in the CNCM I 4970 group samples, suggesting that this strain not only activated type 2 immunity but also the Th17 response. This was not the case for CNCM I 4969.

The administration of CNCM I 4969 or CNCM I 4970 in SPF mice did not change the frequency of lung DC and T cells observed after HDM challenge (data not shown, one experiment). The mechanism underpinning the CNCM I 4969 protective effects requires further investigations. When we inoculated CNCM I 4969 during the HDM challenge (d8) instead of before the sensitization (d2), we lost the protective effect of the strain (Supplementary Figure S4). We believe the timing for efficient CNCM I 4969-driven

attenuation to be crucial (Supplementary Figure S4). Our observations are in accordance with early life as a high susceptibility period for bacterial priming of the lung. The exploration of the influence of microbiota on lung immunity and the potential dysbiosis of microbial communities constitute a new and promising area of research, which will have an impact on the prevention and management of pulmonary diseases such as asthma.

## Conflict of Interest

The authors declare no conflict of interest.

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## Author contributions

Conceptualization: AR, BL, PL, HH and MT; methodology: AR and AB; investigation: AR, DD, MLN, AB, VR, EM and MT; writing—original draft: AR and MT; writing—review and editing: AR, DD, SR, BL, PL, HH and MT; funding acquisition: AR, BL, PL, HH and MT; resources: AB, BL, SR, PL, HH and MT; supervision: AR, HH and MT; project administration: AR, HH and MT.

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# Paradigms of Lung Microbiota Functions in Health and Disease, Particularly, in Asthma

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Improvements in our knowledge of the gut microbiota have broadened our vision of the microbes associated with the intestine. These microbes are essential actors and protectors of digestive and extra-digestive health and, by extension, crucial for human physiology. Similar reconsiderations are currently underway concerning the endogenous microbes of the lungs, with a shift in focus away from their involvement in infections toward a role in physiology. The discovery of the lung microbiota was delayed by the long-held view that the lungs of healthy individuals were sterile and by sampling difficulties. The lung microbiota has a low density, and the maintenance of small numbers of bacteria seems to be a critical determinant of good health. This review aims to highlight how knowledge about the lung microbiota can change our conception of lung physiology and respiratory health. We provide support for this point of view with knowledge acquired about the gut microbiota and intestinal physiology. We describe the main characteristics of the lung microbiota and its functional impact on lung physiology, particularly in healthy individuals, after birth, but also in asthma. We describe some of the physiological features of the respiratory tract potentially favoring the installation of a dysbiotic microbiota. The gut microbiota feeds and matures the intestinal epithelium and is involved in immunity, when the principal role of the lung microbiota seems to be the orientation and balance of aspects of immune and epithelial responsiveness. This implies that the local and remote effects of bacterial communities are likely to be determinant in many respiratory diseases caused by viruses, allergens or genetic deficiency. Finally, we discuss the reciprocal connections between the gut and lungs that render these two compartments inseparable.

**Keywords:** lung, gut, microbiota, physiology, asthma, immunity, gut-lung axis

**Abbreviations:** AMPs, antimicrobial peptides; AMs, alveolar macrophages; BAL, bronchoalveolar lavage; CCL11, C-C motif chemokine 11; CFU, colony-forming unit; CLR, C-type lectin receptor; CO<sub>2</sub>, carbon dioxide; DC, dendritic cell; DNA, deoxyribonucleic acid; FOXP3, forkhead box P3; GF, germ-free; HDM, house dust mite; HFD, high-fat diet; IFN $\gamma$ , interferon gamma; Ig, immunoglobulin; IL, interleukin; iNKT, invariant natural killer T cells; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; NLR, NOD-like receptor; PAR, protease-activated receptor; PD-1, programmed cell death protein 1; pDCs, plasmacytoid dendritic cells; PD-L1, programmed death-ligand 1; PRRs, pattern recognition receptors; rRNA, ribosomal ribonucleic acid; SCFAs, short-chain fatty acids; SFB, segmented filamentous bacteria; SPE, specific pathogen-free; Th, T-helper; TLR, Toll-like receptor; T<sub>reg</sub>, regulatory T cell.

## PAUCITY AND CONTINUAL RENEWAL: TWO MAIN CHARACTERISTICS OF THE LUNG MICROBIOTA

The lung microbiota has a low density, at  $10^3$ – $10^5$  CFU/g of lung tissue, as estimated by culture methods, in mice (Remot et al., 2017). Human lungs harbor approximately  $2.2 \times 10^3$  bacterial genomes per  $\text{cm}^2$  (Hilty et al., 2010). The maintenance of a small bacterial community in the lungs seems to be a hallmark of good health. The microbial population of the lung is smaller than that of the colon, which is one of the most densely populated ecosystems in the body, with a microbiota of up to  $10^{11}$  CFU/g of luminal content. However, the microbial population of the lung is equivalent to that of the duodenum (around  $10^4$  micro-organisms per mL of content) (Aron-Wisniewsky et al., 2012).

The micro-organisms comprising the microbiotas of both the gut and lungs enter the body via the oral cavity. Bacteria travel to the lungs suspended in air and on microparticles in secretions, such as saliva, whereas the bacteria colonizing the intestine may also be present in ingested food. The lung microbiota disperses from the oral cavity, and a constant balance is maintained between microbial immigration and elimination (Morris et al., 2013; Dickson and Huffnagle, 2015; Venkataraman et al., 2015; Figure 1A). The immigration of micro-organisms results from mucosal dispersion, micro-aspiration, and inhalation (Dickson and Huffnagle, 2015; Segal et al., 2016). Humans breathe through both the nose and mouth, whereas mice are obligate nasal breathers (Singh et al., 2017). Anatomical features and natural modes of breathing influence the arrival of microbes in the lung. The elimination of micro-organisms is governed by mucociliary movements, coughing, and host immunity. During lung disease, the balance between immigration and elimination is disturbed, resulting in alterations to the lung microbiota, with bacteria displaying competitive advantages becoming predominant (Dickson and Huffnagle, 2015; Figure 1B). Venkataraman et al. (2015) has suggested that the degree of departure from neutrality is correlated with disease severity in the lung. “Neutrality” refers to the neutral biodiversity theory, according to which, all micro-organisms have similar opportunities of reaching and growing in a specific environment, but also of being lost from that environment. In this model, the microbial communities are not selected by the resources accessible in the environment or the inter-species interactions leading to the creation of multiple niches in an environment (Venkataraman et al., 2015). In healthy conditions, the lung microbiota disperses neutrally from the mouth, whereas lung diseases are associated with stronger selection for specific microbes. The lung microbiota seems to be shaped by continual waves of intrusion and expulsion in healthy humans, because the installation of dominant bacterial communities tends to be restricted to pathological contexts. The overgrowth of bacterial species, leading to a decrease in the species richness of the lung microbiota, is associated with the progression of diseases such as cystic fibrosis and with infections (de Dios Caballero et al., 2017; Zemanick et al., 2017).

The low density and continual renewal of the lung microbiota are not inconsistent with a major impact on respiratory health and homeostasis. The duodenal microbiota plays a key role in iron uptake and storage in the context of digestive physiology (Deschemin et al., 2016). The transit of bacteria through a tissue may have a long-term impact on immunity, as shown for the gut (Hapfelmeier et al., 2010; Tomas et al., 2013). A low density and continual renewal are, thus, intrinsic properties of the microbial stimulation of the lungs that may modify lung immunity and physiology.

## COMPOSITION OF THE LUNG MICROBIOTA

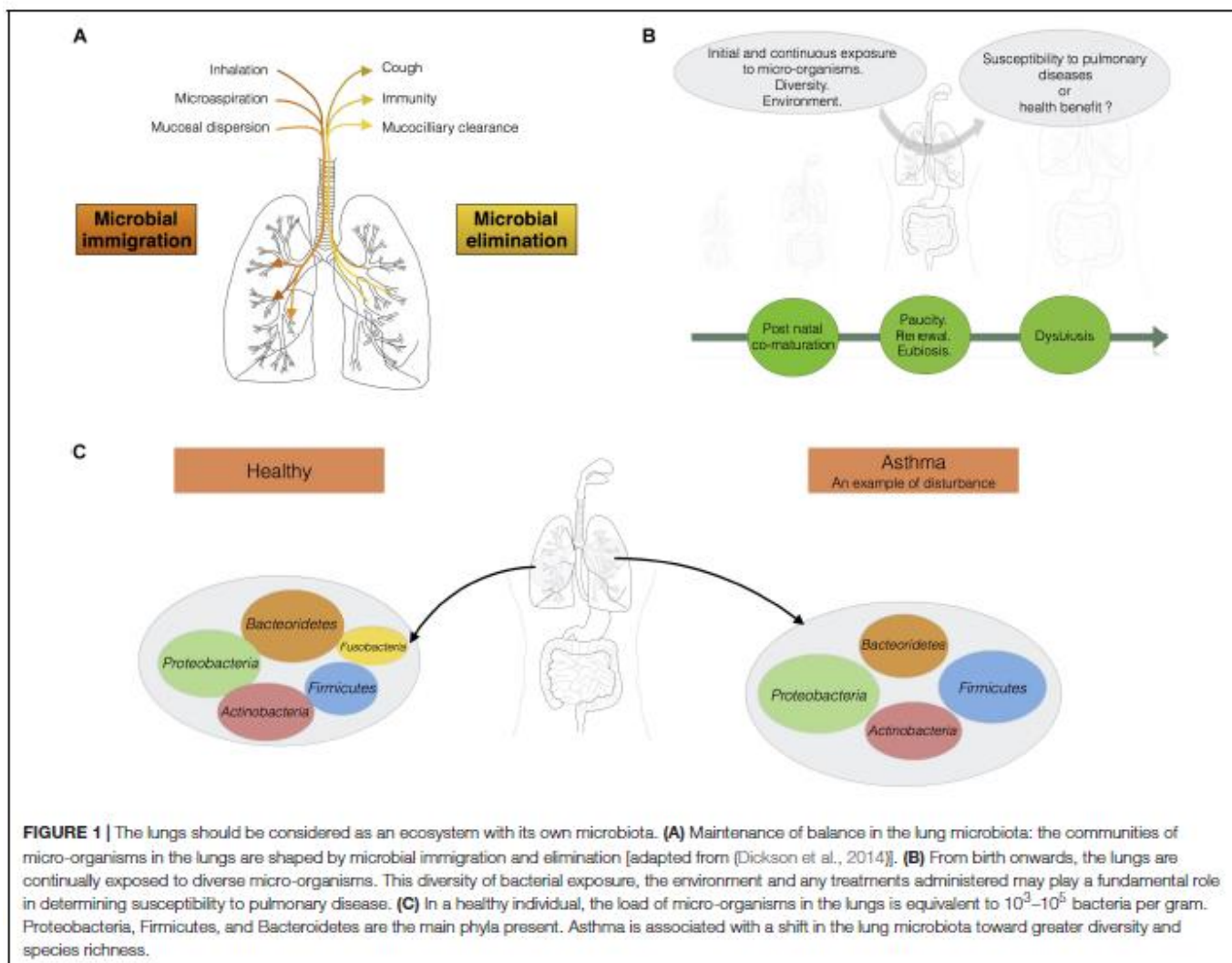
The microbial community is continually being renewed and replaced, but most of the microbes involved in these fluxes belong to four phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Dickson and Huffnagle, 2015; Segal et al., 2016; Yu et al., 2016; Figure 1C). In healthy individuals, *Prevotella*, *Streptococcus*, *Veillonella*, *Neisseria*, *Haemophilus*, and *Fusobacterium* are the most abundant genera in the lungs (Hilty et al., 2010). The four main phyla present are identical in humans and mice. However, Bacteroidetes and Firmicutes predominate in humans, whereas Proteobacteria and Firmicutes predominate in mice. The respiratory microbiota has also been described in domestic animals (cats, dogs), and in farm animals (pigs, sheep, and calves), which can serve as relevant translational models for humans (Ericsson et al., 2016; Glendinning et al., 2016; Nicola et al., 2017; Siqueira et al., 2017; Vientos-Plotts et al., 2017a).

The lung microbiota displays greater spatial variation between than within individuals, and differences between sites in the lung (position relative to the alveoli) result from waves of elimination/immigration and differences in distance from the mouth, which serves as the source of the community (Dickson and Huffnagle, 2015). The analysis of low-density communities is a methodological challenge. In low-density samples, contaminant (or non-related) DNA can predominate over the true sample DNA, creating a shift in the microbial profile obtained. A major impact of extraction methods on relative abundance and bacterial representation has been reported at densities below  $10^6$  bacteria per mL of sample (Biesbroek et al., 2012). The analysis of low-density communities can be challenging, and bias is likely, so particular attention must be paid to the choice of the method and data interpretation, particularly for the lung microbiota.

Due to the high degree of variability between individuals, there is currently no consensus concerning the definition of a “typical” microbiota, constituting a state of homeostasis between the microbiota and the host cells. Moreover, it remains unclear whether specific bacteria or microbiota profiles could serve as markers or drivers of good lung health. There are probably beneficial lung bacteria, as already suggested in the intestine for commensal organisms such as *Faecalibacterium prausnitzii* (Miquel et al., 2015).

During lung diseases, such as asthma in particular, a shift in the lung microbiota is observed that may be seen as an





imbalance or dysbiosis (Hooks and O'Malley, 2017). This shift in the lung microbiota may also be interpreted as the emergence of particular dominant bacteria in lungs. It remains a matter of debate whether we should be talking about dysbiosis, stable colonization, or infections of the lungs. The function and causal role of this dysbiosis in the onset and outcome of asthma remain unclear. An analysis of BAL from children with severe asthma has shown a phylum distribution different from that in control subjects, with, in order of abundance, Proteobacteria, Firmicutes (mainly *Streptococcus*), Bacteroidetes (mainly *Prevotella*), and Actinobacteria (Hilty et al., 2010). At genus level, *Staphylococcus* and *Haemophilus* are more abundant in asthma sufferers, whereas *Prevotella* is more abundant in controls (Hilty et al., 2010). The lung microbiota is more diverse and abundant in some subjects with asthma (Hilty et al., 2010; Huang et al., 2011; Goleva et al., 2013; Fujimura and Lynch, 2015). As for all microbial communities, measurements of abundance and diversity will improve our understanding of the ecological mechanisms underlying health and the management of endogenous communities (Shade, 2017). We now need to determine the mechanisms underlying the maintenance of lung

microbial communities, to find ways of preventing respiratory diseases, such as asthma.

## THE PHYSIOLOGICAL CHARACTERISTICS OF THE LUNGS INFLUENCING THE HOMEOSTASIS BETWEEN THE LUNG AND ITS MICROBIOTA

Microbial immigration and elimination govern the composition of a healthy lung microbiota, but, conversely, certain physiological features of the respiratory tract may favor the installation of a dysbiotic microbiota, influencing susceptibility to pulmonary diseases. The main function of the lungs is to transfer oxygen from the air into the bloodstream, in exchange for  $\text{CO}_2$ . The action of the diaphragm increases lung volume, decreasing pressure in the lung and causing air to enter (Figure 2). Temperature varies along the respiratory tract, from the mouth and nose to the alveoli. The respiratory system



gradually warms the air to 37°C. The gradients of pressure and temperature between the upper respiratory tract and the alveoli may affect bacterial communities. The pulmonary epithelium is composed of ciliated and secretory cells, but is not continuous from the upper respiratory tract to the alveoli (Evans et al., 2010; **Figure 2**). Indeed, in the large bronchi, the mucous and serous cells are located in a submucosal gland that produces mucus (not shown in **Figure 2**). Moving toward the bronchiole, mucus is produced by club and goblet cells. Type I and II pneumocytes form the alveolar epithelium, which secretes a surfactant rather than mucus. Mucus is a gel consisting mostly of water and complex polysaccharides, such as mucins (Evans et al., 2010). MUC5AC (from goblet cells) and MUC5B (from submucosal glands) are the dominant mucins in human airways, together with MUC2, which is produced in only small amounts (Proud and Leigh, 2011). Water and mucins form a thin mobile layer that is supported by a periciliary layer covering the cilia. In a healthy individual, the mucus layer provides an effective defense against epithelial injury, but excessive mucus production contributes to obstruction in several respiratory diseases (e.g., pneumonia, asthma, chronic obstructive pulmonary diseases, cystic fibrosis).

This obstruction may lead to the production of even more mucus, making it increasingly difficult for the cilia to transport the mucus out of the lungs. A longer residence time of mucus in the airways may favor the selection of certain bacteria with a high tropism for mucus, leading to the installation of pathogens (Flynn et al., 2016). Flynn et al. (2016) have shown that some bacteria present in sputum make use of mucins to produce metabolites, such as propionate in particular, which can be used by *Pseudomonas aeruginosa*. The maintenance or selection of the microbiota is also determined by the nutrient sources available in a particular ecological niche. In the gastrointestinal tract, nutrient sources capable of supporting microbial growth are present at high abundance (due to the breakdown of food). The microbes of the intestinal tract are, therefore, commensal, because they can share the food we eat. By contrast, most of the nutrients available in the lungs are derived from host compounds, such as Igs, cytokines, defensins, lactoferrins, and mucins (Flynn et al., 2016). Lung epithelial cells express various innate sensors on their membranes and in their cytoplasm (TLR, NLR, CLR, and PAR) (Jung et al., 2016) that can detect microbes and activate molecular cascades in host cells, triggering the induction of tolerance or inflammation (Hammad et al., 2009; Di Stefano et al., 2017). For example, many studies of the role of TLR4 in asthma have been performed with the TLR4 agonist LPS. Bottomly's group has shown that sensitization to inhaled inert proteins requires LPS and the TLR4 signaling pathway (Eisenbarth et al., 2002). The dose of LPS is critical, as low doses break tolerance and exacerbate the signs of asthma (Eisenbarth et al., 2002), whereas high doses prime protective responses (Hammad and Lambrecht, 2008; Rodriguez et al., 2014). These differences in lung biotic (cell layers) and abiotic (temperature, pressure, mucus, surfactant) environments may have a major impact on the installation and location of bacterial communities, particularly if they lead to certain bacteria being selected and becoming predominant in disease processes.

## PROGRESSIVE AND SEQUENTIAL INSTALLATION OF THE MICROBIOTA IN THE LUNGS AFTER BIRTH

### Effect of Delivery Mode on the Lung Microbiota

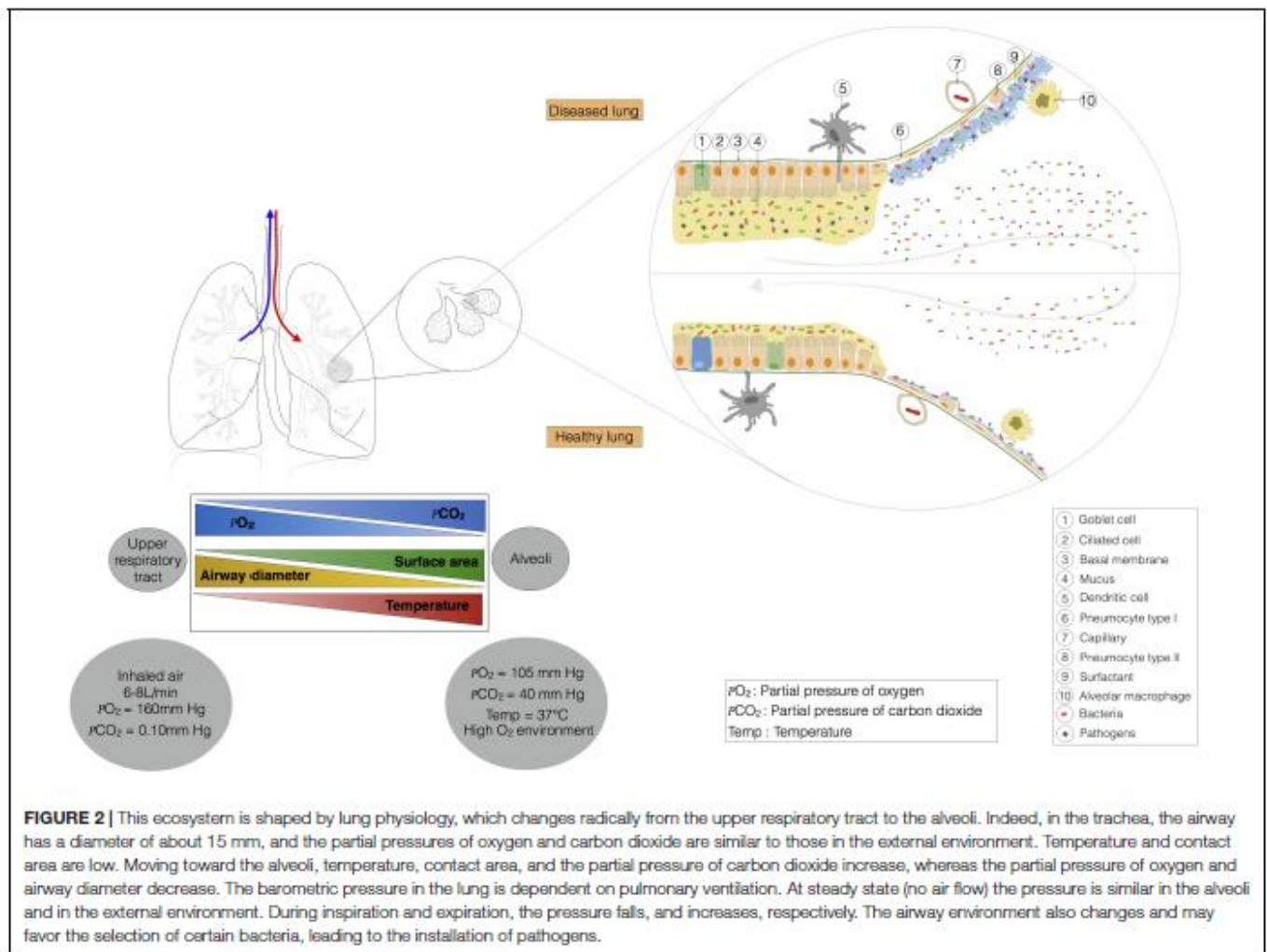
The airway microbiota sampled by tracheal aspiration is similar in preterm infants born by Cesarean section and in those born by the vaginal route, and consists predominantly of Proteobacteria and Firmicutes during the first few days of life (Lal et al., 2016). However, despite the limited impact of the mode of delivery on the composition of the nasopharyngeal microbiota immediately after birth, subtle differences in respiratory microbial development may appear over time between children born by the vaginal route and those delivered by Cesarean section (Bosch et al., 2016). It therefore remains unclear whether mode of delivery has a strong or weak influence on the composition of the lung microbiota in babies (Blaser and Dominguez-Bello, 2016; Chu et al., 2017), but the microbiota of the mouth and, by extension, the lungs, may be subtly influenced by delivery conditions.

### Post-natal Co-maturation of the Microbiota and Lungs

The lungs of newborn humans face daily challenges in the form of diverse new microbes and environmental components, including allergens (**Figure 1B**), and the postnatal period has a major impact on future health. The development of the lungs, like that of other organs, is not complete at birth. The lungs begin to develop, with the formation of the branching structure, during the embryonic and fetal periods. Lung development is then completed during the postnatal period, when the terminal units of the branching structure, the alveoli, finish developing, along with the vascular system. The immunological development of the lungs also follows a chronological pattern, beginning in the embryo and continuing through the post-natal period, with the sequential arrival of monocytes/macrophages and granulocytes/neutrophils, the recruitment of type 2 innate cells and the accumulation of DC, B, and T cells until weaning (Drajac et al., 2017). Biesbroek et al. (2014) analyzed the development of the microbiota in the upper respiratory tract (nasopharyngeal samples) at different time points during the first 2 years of life (1.5, 6, 12, and 24 months) in healthy individuals. They revealed different microbiota profiles between the members of the cohort at ages as young as 1.5 months. Early colonization with specific micro-organisms influences the subsequent stability of the upper respiratory tract microbiota and susceptibility to pulmonary infection (Biesbroek et al., 2014).

Singh et al. (2017) analyzed the sequential arrival of bacteria in the lungs of mice aged between one and 8 weeks. They observed dynamic changes in mouse lungs over this period. For example, the genus *Streptococcus* predominated when the mice were 2 weeks old, whereas *Lactobacillus* and *Achromobacter* were the most abundant genera when the mice were 4 weeks old (Singh et al., 2017). The phyla Firmicutes and Gammaproteobacteria





**FIGURE 2** | This ecosystem is shaped by lung physiology, which changes radically from the upper respiratory tract to the alveoli. Indeed, in the trachea, the airway has a diameter of about 15 mm, and the partial pressures of oxygen and carbon dioxide are similar to those in the external environment. Temperature and contact area are low. Moving toward the alveoli, temperature, contact area, and the partial pressure of carbon dioxide increase, whereas the partial pressure of oxygen and airway diameter decrease. The barometric pressure in the lung is dependent on pulmonary ventilation. At steady state (no air flow) the pressure is similar in the alveoli and in the external environment. During inspiration and expiration, the pressure falls, and increases, respectively. The airway environment also changes and may favor the selection of certain bacteria, leading to the installation of pathogens.

arrive in the lungs before Bacteroidetes (Gollwitzer et al., 2014). Viable bacteria begin to arrive in the lungs of mice after birth (Remot et al., 2017), and the number of pulmonary bacteria significantly increases until weaning and adulthood (Gollwitzer et al., 2014; Remot et al., 2017). The diversity and abundance of cultivable bacteria also increases with age, from birth to adulthood, in mice (Remot et al., 2017; Singh et al., 2017). Most descriptions of the progressive installation of the lung microbiota after birth relate to mice, but this pattern is consistent with the overall maturation of human microbiotas, which is particularly well described for the gut, with a progressive acquisition of diversity and stability over the first 3 years (Lozupone et al., 2012).

### Hygiene Theory and Asthma

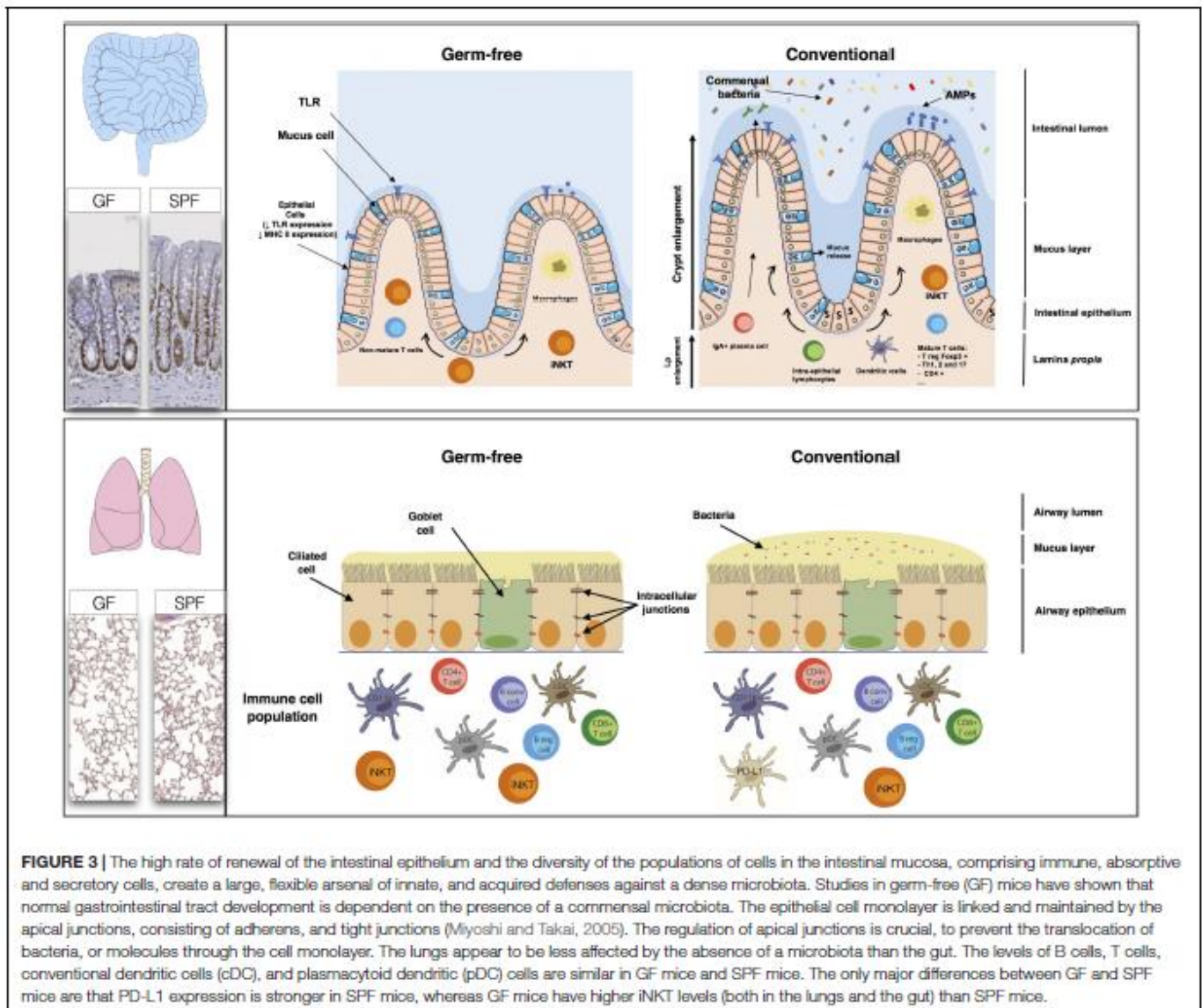
According to the hygiene theory, lower levels of exposure to microbes in urban than in rural areas result in a higher incidence of allergy and asthma (Olszak et al., 2012; Marsland, 2013; Segal et al., 2014). Exposure to LPS, a component of Gram-negative bacteria, decreases asthma levels in mice by suppressing the activation of epithelial and DCs via induction of the ubiquitin-modifying enzyme A20 (Schuijs et al., 2015). Several cross-sectional studies in different countries have compared

the prevalence of asthma, hay fever and allergic sensitization in children living in farming and non-farming environments (Kilpelainen et al., 2000; Riedler et al., 2000; Portengen et al., 2002; Douwes et al., 2007). They showed that children born and raised in a farming environment were less prone to the development of atopic symptoms and asthma later in life. This protective effect was even stronger in adults that had remained in the farming environment (Douwes et al., 2007). These observations support the hypothesis that exposure to a wide range of diverse microbial signals during the first few months of life has a major impact on susceptibility to the development of asthma.

### IMPACT OF THE LUNG MICROBIOTA ON THE ADAPTIVE AND INNATE IMMUNE CAPACITIES OF THE LUNGS

The lung may display a similar homeostasis to the gut, in terms of the co-evolution of eukaryotic and prokaryotic cells, and dialog between these cells. In the gut, the microbiota is involved in digestion, energy provision, maturation of the immune system





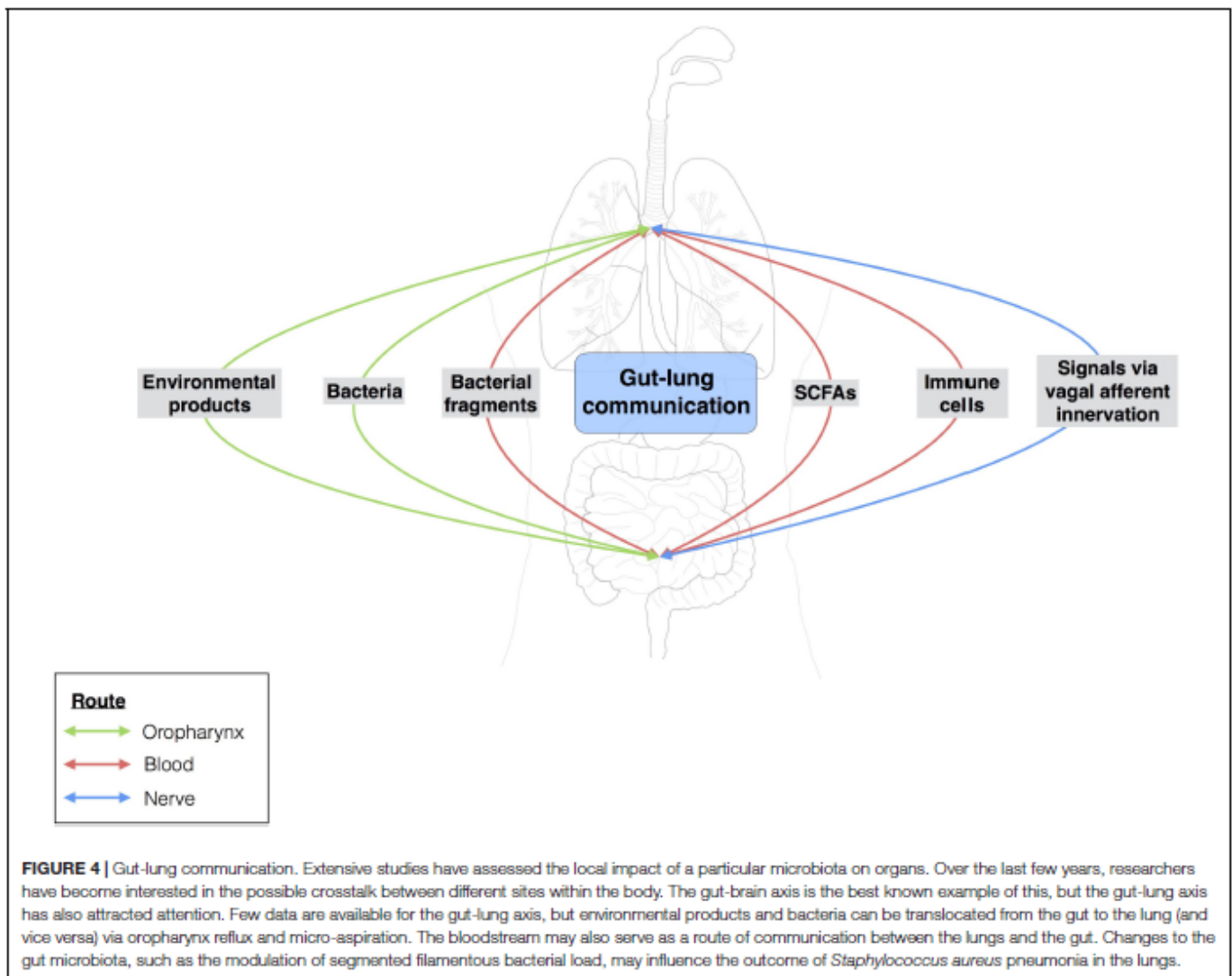
and shaping the structure and modulating the absorption and secretion functions of the epithelium. However, much less is known about the physiological effects of the lung microbiota (Figure 3).

## Immune System

Many studies on the gut have reported changes to the immune phenotype, with deficits of both the innate and adaptive immune components of the intestinal mucosa in GF mice. Several bacterial species have been shown to have different modulatory effects on the host immune system, highlighting the need for specific bacteria within a given developmental window for the normal patterning of host immunity (Olszak et al., 2012; Ubags and Marsland, 2017). GF mice have a thinner lamina propria, with fewer resident mature immune cells, and the presence of a complex microbiota triggers the proliferation, differentiation, and maturation of immune cells, leading to an increase in microbiota-selected IgA<sup>+</sup> (immunoglobulin A) plasma cells or

T-cell subsets, such as T<sub>reg</sub> Foxp3, Th1 (T-helper), Th2, and Th17 lymphocytes (Macpherson and Uhr, 2004; Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Some of these mechanisms are mediated by receptors, such as TLRs, which are essential for homeostasis in the intestinal epithelium (Rakoff-Nahoum et al., 2004).

Studies comparing GF and SPF mice during the first few weeks of life have shown that microbial colonization of the lung has no major effect on the subsets of immune cells present. The levels of B and T (CD4 and CD8) cells, conventional CD11b<sup>+</sup> and CD103<sup>+</sup> DCs and pDCs are similar in the presence and absence of a microbiota (Gollwitzer et al., 2014; Remot et al., 2017). However, the lungs of GF mice contain 2.5 times as many iNKT than those of SPF mice. Interestingly, when the lungs of GF neonates were exposed to a conventional microbiota, iNKT cell levels were found to be similar to those in SPF mice (Olszak et al., 2012). The bacterial communities in the lung modulate the expression of certain innate immunity genes,



resulting in higher levels of IL-5 (interleukin), IL-10, IFN $\gamma$ , and CCL11 in SPF mice. The level of expression of PD-L1 on CD11b<sup>+</sup> DCs and the frequency of FoxP3<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells are also higher in the lungs of SPF neonates (Gollwitzer et al., 2014). Comparisons of SPF, GF, conventional, and antibiotic-treated rodents can provide information about the modification of pattern recognition receptor expression (PRRs: TLR, NLR, CLR, and PAR) in the lungs by the microbiota. Segal et al. (2016) recently showed that the TLR4 responses of AMs were influenced by the composition of the lung microbiome.

## Mucus

Germ-free mice have a thinner mucus layer in the gut than conventional mice with a complex microbial ecosystem impregnating the mucus layer close to the epithelial cells and AMPs. These small peptides keep bacteria off of the epithelium and limit bacterial growth. AMP production may be modulated in a specific manner by the microbiota, as reported for beta-defensins, or may be microbiota-independent, as described for lysozymes (Pütsep et al., 2002; Gallo and Hooper, 2012). Mucus

production is a dynamic process that may be accelerated or slowed by the microbiota, as shown in the intestine (Wrzosek et al., 2013). Few data are available for the lung, but *muc5ac* (the main mucin in the lung) mRNA levels have been shown to be higher in SPF mice than in their GF counterparts (Remot et al., 2017). Yun et al. (2014) have also reported lower levels of mucus production by the lungs when bacteria are absent or present at low abundance. The production of mucus by the lungs therefore appears to be shaped by the lung microbiota, through as yet unknown mechanisms. By modulating mucus production, the microbiota may modify the barrier function of the respiratory epithelium or favor invasion by mucus-degrading bacteria in some diseases.

## Tissue Organization

As illustrated in Figure 3, microbes can greatly modify the morphology of the intestinal epithelium (Cherbuy et al., 2010; Jones et al., 2013; Tomas et al., 2013), with some commensal strains of *Escherichia coli* having morphogenic activity (Tomas et al., 2015). The morphogenic effects of the microbiota



are more subtle in the lungs. No marked differences in pulmonary structure, epithelium thickness, or bronchus number are observed between lungs with and without a microbiota, but the number of alveolae is greater in the presence of a microbiota (Yun et al., 2014; Remot et al., 2017).

## Tolerance

The differences (in the immune system, mucus, and epithelium) between GF and SPF animals are less marked in the lungs than in the gut, but microbes in the lung clearly modulate susceptibility to respiratory disorders. During their development, the lungs of neonates are exposed to bacterial stimuli that may affect the maturation of the pulmonary tissue, conferring susceptibility to lung disorders, such as allergic asthma (Sabatel et al., 2017; Ubags and Marsland, 2017). Lung microbes have been shown to promote tolerance, potentially accounting for the hypersensitivity of neonates to allergens. Indeed, during the first 2 weeks of life, mice display high levels of allergic airway inflammation, producing large amounts of IL-4, IL-5, and IL-13 following treatment with HDM allergen. The overproduction of these Th2 cytokines in the lungs of neonates after HDM treatment is coupled to an increase in the proportion of FoxP3<sup>+</sup>CD25<sup>+</sup>T<sub>reg</sub> and CD11b<sup>+</sup> DCs and an increase in expression of the surface ligands PD-L1, PD-L2, and CD40. This allergic airway inflammation is significantly attenuated in adult mice. Gollwitzer et al. (2014) suggested that the tolerance of mice to HDM allergen might improve through the expression of PD-1 on lung T<sub>reg</sub> cells, together with its ligand PD-L1 on CD11b<sup>+</sup> DCs. The postnatal susceptibility period strongly affects responsiveness to aeroallergens. It is also correlated with the installation of the stable microbiota in the lungs and the final stages of pulmonary development. Our work has helped to demonstrate that the administration of primary lung-colonizing strains before HDM treatment affects responsiveness to aeroallergens in mice (Remot et al., 2017). Microbial stimulation of the lung at a given time may, therefore, promote allergen tolerance. The impact of the lung microbiota on lung immune capacity in this window of opportunity thus appears to play a key role in susceptibility to the development of allergic diseases, such as asthma, in adulthood.

## THE GUT-LUNG AXIS

The gut-lung axis comprises the anatomical, systemic, and nervous system connections mediating reciprocal exchanges of microbial signals between the lungs and the gut (Figure 4). One of the connections between the gut and the lung involves the translocation of bacteria via oropharynx reflux. Indeed, the human body experiences multiple reflux events (especially in pathological conditions) that can transport different bacterial communities from the digestive tract to the upper respiratory tract, with the bacteria then translocated to the lungs by micro-aspiration. However, this does not mean that bacteria from the digestive tract can reside in the lungs. Moreover, bacteria and bacterial fragments may also be translocated in the blood and lymph. The blood and lymph play a major role in the migration of immune cells to distal sites. For example, bacteria from the gut

taken up into DCs and macrophages through phagocytosis prime naïve B and T cells, which may then migrate to the lungs or return to the gut (Bingula et al., 2017).

In physiological conditions, the gut microbiota of old mice (18–22 months of age) may influence inflammation in the lungs and the immune senescence of macrophages (Thevaranjan et al., 2017). High levels of circulating bacterial toxins after fecal transplantation have also been shown to result in low levels of tight junction gene expression and lethal pulmonary damage (Ji et al., 2014). The bacterial taxa implicated are mostly clostridial species. The existence of these connections was suggested by numerous epidemiological observations, but the underlying mechanisms linking gut and lung physiology remain elusive and hypothetical.

Short-chain fatty acids, which are produced in large amounts by some commensal bacteria, can act as substrates for host cells and as signaling molecules between tissues. The metabolic profiling of the microbial community of the lungs is incomplete and the role of SCFAs as organizers of endogenous lung microbial communities, local actors in the respiratory epithelium and immunity, and systemic mediators remains unclear. The importance of these bacterial metabolites and bacteria in the gut for host local immunity has been studied in detail. However, little is known about their impact on distal immunomodulation, in the lungs. For example, few data are currently available concerning immunomodulation by the microbiota at distal sites. However, one study has shown that mice lacking SFB in the gut are prone to more severe *Staphylococcus aureus* pneumonia. The bacterial load in the lungs of mice lacking SFB is 21 times higher than that of mice with SFB in the gut. This higher bacterial load is accompanied by a modulation of pulmonary Th17 immunity, with lower levels of IL-22 in BAL fluid (Gauguet et al., 2015). Modulation of the composition of the gut microbiota in mice regulates the immune response of the respiratory tract and alters susceptibility to pulmonary influenza infections (Ichinohe et al., 2011; Rosshart et al., 2017).

A recent review described a pathogenic link between the microbiota and the gut-lung axis (Budden et al., 2017). We will therefore focus here on the gut-lung axis in asthma. It has been shown that the progressive and sequential acquisition of the gut microbiota after birth determines subsequent susceptibility to allergy (Arrieta et al., 2015; Fujimura et al., 2016; Stokholm et al., 2018). During the perinatal period, the gut microbiota plays a key role in determining future tolerance, the maturation of immunity and asthma risk. It is not yet possible to estimate the relative contributions of the establishment of the lung and gut microbiotas in humans. The gut-lung axis may also be affected by the dual location of some environmental allergens, weakening the barriers in both the gut and the lung and stimulating a cascade of detrimental inflammatory pathways. In particular, HDM is both inhaled and ingested, and its Der p1-associated allergen is detected in the gut, where it can impair the barrier function of the intestine (Tulic et al., 2016). The manipulation of both gut and lung microbiotas, by oral supplementation or nasal administration, may be beneficial, favoring homeostatic feedback and decreasing risk (Arrieta et al., 2015; Remot et al., 2017; Durack et al., 2018). Many studies have tested the hypothesis that



pre- and probiotics can protect against asthma, but the possible mode of action has yet to be elucidated in mice. The use of a large-animal model to study the effects of oral probiotics on respiratory microbiota may be of interest as a potential translational model for humans (Vientos-Plotts et al., 2017b). A few preclinical data for humans supporting the use of specific products are available (Mennini et al., 2017). Conversely, specific diets may also increase the risk. In mice a HFD, and the consumption of saturated fatty acids (palmitic acid) in particular, has been shown to increase the proportion of circulating monocytes and AMs in the lung. Moreover, the combination of a HFD with HDM stimulation results in airway responsiveness, inflammatory cell levels, goblet cell hyperplasia, total cell number, levels of neutrophils, eosinophils, and macrophages and of IL-13, IL-17A, and IL-1 $\beta$  production greater than those in mice fed a HFD in the absence of HDM stimulation (Tashiro et al., 2017). Marsland's group demonstrated that the metabolism, by the gut microbiota, of dietary fiber influences allergic airway disease and haematopoiesis (Trompette et al., 2014). Mice fed a high-fiber diet had high levels of SCFA in the blood and were protected against HDM-induced asthma, whereas a low-fiber diet resulted in lower SCFA levels and a higher frequency of asthma. Protection was associated with the beneficial effects of propionate, which modified haematopoiesis in a GRP41-dependent manner, particularly in terms of DC levels and Th2 differentiation. Similar correlations between a low incidence of asthma and changes in the intestinal microbiota after fiber intake have been reported in humans (De Filippo et al., 2010; Marsland et al., 2015).

There is currently no consensus definition of a "healthy" lung microbiota as a function of age, diet, or environment and, given the considerable interindividual variability of the gut microbiota, we are still far from a definition of the best gut/lung microbiota configuration to optimize digestive and respiratory health. It should also be noted that the influence of the gut microbiota on distal immunity is not restricted to the lung. The gut microbiota has been shown to affect hepatic

immune responses (Chou et al., 2015), and to shape the immune pancreatic environment (Ryu and Stappenbeck, 2015). All these studies support the hypothesis that the host microbiota can affect the "common mucosal immune system."

## CONCLUSION

Interest in the lung microbiota has steadily increased over the last decade, and it is now widely accepted that the lungs harbor bacterial communities. Like those of the gut, the microorganisms of the respiratory microbiota play a role in health and diseases, such as asthma. We are gradually learning more about the densities of the various members of the community, their sources and their dispersion throughout the branched structure of bronchi. More intensive studies of the local impact of the lung microbiota and the pathways involved in gut-lung communication are required. A number of questions remain to be addressed. Are bacteria or their products translocated via the oropharynx reflux or via the blood in pathogenic and physiological conditions? How intense are immune cell exchanges between the two sites, and what impact do they have? What do lung bacteria use as an energy source? What reciprocal impacts do lung bacteria and eukaryotic cells have in the lungs and other tissues? Whatever the answers to these questions, the gut and lung ecosystems (consisting of micro-organisms and host cells) clearly link nutrition, respiratory and digestive health and immune defenses, through an intricate system of reciprocal communication.

## AUTHOR CONTRIBUTIONS

EM, UE-V, AR, and MT conceived the work and made major contributions to the writing of the manuscript. EM and UE-V made major contributions to the illustrations. DD, CC, PL, and SR contributed to the writing of the manuscript.

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# Neutrophils and Close Relatives in the Hypoxic Environment of the Tuberculous Granuloma: New Avenues for Host-Directed Therapies?

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb) is one of the most prevalent lung infections of humans and kills ~1.7 million people each year. TB pathophysiology is complex with a central role played by granuloma where a delicate balance takes place to both constrain bacilli and prevent excessive inflammation that may destroy lung functions. Neutrophils reach the lung in waves following first encounter with bacilli and contribute both to early Mtb elimination and late deleterious inflammation. The hypoxic milieu where cells and bacilli cohabit inside the granuloma favors metabolism changes and the impact on TB infection needs to be more thoroughly understood. At the cellular level while the key role of the alveolar macrophage has long been established, behavior of neutrophils in the hypoxic granuloma remains poorly explored. This review will bring to the front new questions that are now emerging regarding neutrophil activity in TB. Are different neutrophil subsets involved in Mtb infection and how? How do neutrophils and close relatives contribute to shaping the granuloma immune environment? What is the role of hypoxia and hypoxia induced factors inside granuloma on neutrophil fate and functions and TB pathophysiology? Addressing these questions is key to the development of innovative host-directed therapies to fight TB.

**Keywords:** neutrophils, *Mycobacterium tuberculosis*, granuloma, lung, HIF, hypoxia, host-directed therapies

## INTRODUCTION

Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) is present worldwide. With estimated 10.4 million new cases and 1.7 million deaths in 2016<sup>1</sup>, TB remains one of the most devastating respiratory disease of human kind. The key cell in Mtb lung infection is the lung alveolar macrophage (AM) that engulfs the bacilli and orchestrates the adaptive host immune response if bacilli are not eliminated (1). This is the starting point for the granuloma, set as an immune defense mechanism that eventually becomes the pathologic signature of Mtb infection. AM plays major roles in the battle between Mtb and the host and a large body of the literature is devoted to this key cell. However, mature neutrophils circulate in high numbers in blood and are also sequestered in the lung (2). As

<sup>1</sup><http://www.who.int/gho/tb/en/>



they are present in the early phase of Mtb infection, before the onset of adaptive immunity, they could play important beneficial protective roles [see extensive review in (3)]. In the zebra-fish (ZF) embryo infected with *M. marinum* (Mm) as a surrogate of Mtb infection in mammals, neutrophils come in response to signals sent by Mm-infected dying macrophages (MPs). Neutrophils dispose off Mm-infected MPs by efferocytosis in the nascent granuloma and kill bacilli through NADPH oxidase-dependent mechanisms (4). We and others have shown in resistant mouse models that neutrophils come in two different waves after Mtb infection before and after the onset of adaptive immunity (5, 6). While the first wave was found to phagocytose BCG—the vaccine strain used against Mtb—*in situ* in the lung, the second T-cell dependent wave was seldom associated with bacilli. In response to virulent Mtb, T-cell dependent neutrophils did not control Mtb growth but rather established close contacts with T-cells in the granuloma (6) suggesting their role in regulation of the adaptive immune response. This is in line with their established role in the formation of the structured mature granuloma in mice (7). However, during active TB, it is now consensus that neutrophils are largely responsible for lung destruction (8). They are the most represented cell subset in sputum (9) and drive an interferon-inducible transcriptional signature in blood cells during active TB (10). Several excellent reviews recently covered neutrophils as “good and bad guys” during TB (3, 8, 11, 12). Such opposite roles may depend on several factors including timing and magnitude of neutrophil recruitment or different neutrophils subsets which respective roles in TB remain elusive. Despite the fact that neutrophils are established as key players in the TB granuloma, the impact of hypoxia on their behavior and functions is still poorly explored and we advocate in this review that this should be reconsidered. Moreover, in the granuloma, the influence of the hypoxic milieu on contribution of neutrophils to production of soluble mediators involved in TB pathophysiology needs to be reconsidered. The world is on high demand of host-directed therapies (HDTs) as adjunct to antibiotics to fight against TB and we hope that our mini review will help to design effective strategies by taking into account the impact of hypoxia on neutrophils.

## THE Mtb GRANULOMA IS A PATHOLOGICAL IMMUNOLOGICAL NICHE WHERE NEUTROPHILS PLAY MAJOR ROLE

For a long time, the granuloma has been considered as an uniquely host-driven response, set to constrain Mtb and prevent bacilli dissemination. This view was challenged when, in ZF embryo, virulent Mm was shown to disseminate and establish infection by manipulation of the nascent granuloma and adjacent stromal cells (13). Today, the host-pathogen mutual benefit of the granuloma is still a matter of debate (14, 15), as is the role of neutrophils in this structure. Some confounding interpretations may come from animal models, especially the mouse, most extensively used in TB research. In humans, primary TB leads to caseating granulomas that necrotize over time.

Cavities, allowing Mtb transmission, represent the most severe signature of the disease (16). Human TB granulomas are hypoxic as demonstrated by Positron Emission Tomography-Computed Tomography (PET-CT) scans using hypoxia-specific tracers in patients with active TB (17). In TB susceptible animals such as the rabbit, the guinea pig, and the non-human primate, hypoxic granulomatous lesions develop in the lung (18). By contrast, the resistant mouse lines C57BL/6 and BALB/c that have been extensively used, do not develop necrotizing hypoxic granulomas which brought the quite general belief that mice are not an adequate model for TB pathophysiology studies (19). However, extremely susceptible mice such as C3HeB/FeJ do develop central caseous necrosis in the lung (20) and these lesions are hypoxic (21). A common feature to all TB susceptible animals that develop hypoxic necrotizing granulomas is the abundance of neutrophils (22, 23). Mtb induces necrosis of human neutrophils, which depends on its main virulence factor, the small protein ESAT-6 secreted by Type VII secretion system. Necrosis is driven by neutrophil-derived Reactive Oxygen Species (ROS) and is required for Mtb growth after uptake of infected neutrophils by human macrophages (24). In C3HeB/FeJ mice, neutrophils dying by necrosis or NETosis rather than apoptosis seem to drive the caseous necrosis and liquefaction process (25). The crucial role of neutrophils and the S100A9 inflammatory protein for granuloma formation is demonstrated (26). Therefore, what “adequate” animal models and available pathology studies in humans teach us is that neutrophils and hypoxia are crucial to the development of lung lesions during TB disease.

However, some clarification is needed regarding the definition of neutrophils. These cells have long been considered as an homogenous population based on their polylobed nucleus and a minimal set of markers: in mice, they are defined by flow cytometry as Gr1, CD11b double positive cells or more recently as CD11b, Ly-6C, Ly-6G triple positive cells. In humans, they are still minimally identified as CD11b<sup>+</sup> CD14<sup>-</sup> CD15<sup>+</sup> cells. The picture has become more complex with the description of Myeloid Derived Suppressor Cell (MDSCs), which largely share markers with neutrophils. MDSCs are an immature and heterogeneous population present at homeostasis and accumulating in pathological situations. Originally described in cancers, MDSCs are increasingly characterized in inflammatory diseases (27, 28). MDSCs suppress T cell responses, via different mechanisms, including production of ROS, nitric oxide (NO), or arginase 1 (29). MDSCs are present as two main subsets: monocytic MDSCs and granulocytic MDSCs (Gr-MDSCs). The later display the same morphology and phenotype as *bona fide* neutrophils. They share the Ly-6G or Gr1 markers. Therefore, MDSCs can robustly be distinguished from *bona fide* neutrophils only based on their suppressive function (30). Expansion of granulocytic and monocytic MDSCs is observed in blood of active TB patients and healthy recently exposed contacts (31, 32). This correlates with enhanced L-arginine catabolism and NO production in plasma from active TB patients (33). In resistant (C57BL/6) or susceptible (129S2) strains MDSCs—defined as Gr1<sup>+</sup> cells—are identified in the lung parenchyma during the course of Mtb infection where they suppress T cells (34). They also vigorously ingest Mtb. Interestingly, in susceptible mice, Gr1<sup>+</sup> MDSCs cells accumulate in higher



numbers and phagocytose more bacilli as compared to resistant mice. Therefore, MDSCs could represent a niche for Mtb replication, helping the pathogen to escape the immune system (34). MDSCs are also associated with TB progression and lethality (35). These findings emphasize the potential of MDSCs as targets for immunotherapy. However, most studies using depletion antibodies that target the Gr1 or the Ly-6G surface marker, do not allow today a clear distinction of the role of *bona fide* neutrophils vs. MDSCs in TB pathophysiology. To add to the complexity, the low density neutrophils (LDNs) have recently been described as a new population of neutrophils. LDNs, displaying heterogeneous morphology and containing mature and immature cells, are immunosuppressive via secretion of IL-10 and expression of arginase-1 (36). Interestingly, mature high density neutrophils (HDNs) can switch to LDNs in a TGF- $\beta$  dependent way, and acquire immunosuppressive functions similar to granulocytic MDSCs (37). First described in cancer (37) and pulmonary pathologies (38), LDNs have also been identified in TB and associated to the severity of the disease. Moreover, Mtb is able to convert HDNs to LDNs *in vitro*, suggesting manipulation by Mtb (39). Even though LDNs are not yet considered as granulocytic MDSCs, the largely shared purification procedure, analysis methods and markers between these two cell populations suggest possible overlaps (30). Mtb infection in mice recruits an altered “neutrophil” population defined as “Gr-1<sup>int</sup>/Ly-6G<sup>int</sup>” cells with lower levels of Gr1/Ly-6G as compared to classical neutrophils. These immature cells highly express the CD115 and CD135 markers and inhibit T cell proliferation (35). Whether these cells are distinct from granulocytic MDSCs remains to be clarified.

Outside from the TB research field, recent studies identified neutrophils as potential players in inflammatory angiogenesis. Neutrophils store Vascular Endothelial Growth Factor (VEGF), a key player in the process of angiogenesis, that they may release upon stimulation. By recruiting neutrophils, MIP-1 $\alpha$  and MIP-2 act in an autocrine loop to promote this process. A new CXCR4<sup>high</sup> and CD49d<sup>high</sup> neutrophil subset, displays angiogenic properties via secretion of high concentrations of MMP-9 promoting neovascularization (40). In a model of skin hypersensitization, Tan et al. demonstrated that neutrophil MMP9 and heparanase, targeting distinct domains of the extracellular matrix, cooperate to release diverse VEGF isoforms and influence their bioavailability and bioactivity during inflammatory angiogenesis (41). In mice and humans, CD49d<sup>+</sup> CXCR4<sup>high</sup> VEGFR1<sup>high</sup> neutrophils are recruited specifically in hypoxic ischemic tissues in a VEGFR1 and VEGFR2 dependent way (42). Whether such neutrophils could contribute to formation of the hypoxic TB granuloma remains on open question.

## HYPOXIA-INDUCED-FACTORS ARE MASTER REGULATORS IN Mtb GRANULOMA

The tremendously exciting field of immunometabolism, which links cellular bioenergetics pathways to immune cell functions, brings new views on the fate of the TB granuloma. In

response to inflammatory environment, MPs switch from oxidative phosphorylation—the mitochondrial respiration system that quiescent cells use to generate energy—to aerobic glycolysis. This shift, called the Warburg effect, was discovered in proliferating cancer cells that highly incorporate glucose, that they convert to lactate while producing ATP and cell-building blocks (43). Master regulators of this switch are Hypoxia-Induced-Factors (HIFs), a family of transcription factors that govern cell reprogramming (44). Under normoxia, the enzymes Prolyl Hydroxylase Domains (PHD) and Factor Inhibiting HIF (FIH) repress HIFs via targeted degradation and transcriptional mechanisms. Under low O<sub>2</sub> tension, these enzymes become inactive, HIFs are stabilized and derepressed and activate a transcriptional program to adapt the cell to hypoxia. Other than O<sub>2</sub>, HIFs respond to a variety of environmental factors. HIF1 $\alpha$ <sup>-/-</sup> mice display enhanced Mtb burden and reduced survival (45). This could be linked to HIFs regulating the bactericidal functions of MPs and neutrophils (46). NF- $\kappa$ b, the master regulator of the inflammatory response, regulates transcription of the *hif1a* gene encoding one of the HIF subunits (47). In MPs, LPS binding to TLR4 activates HIF-1 $\alpha$  that upregulates IL-1 $\beta$  production. The signaling occurs through succinate, one intermediate of the tricarboxylic acid cycle (48) that accumulates upon reprogramming of the MP toward aerobic glycolysis. However, this effect cannot be generalized to all TLR- signaling pathways (49). Imaging with glucose tracers illustrates high glucose uptake after infection with Mtb in the lungs of C3HeB/FeJ mice (50) non-human primates (51) and humans (52). Aerobic glycolysis is confirmed by NMR-analysis of metabolites in mice (53) and guinea pigs (54), or global transcriptomics of genes encoding glycolytic enzymes in the lungs of mice, rabbits, and humans (55). Reprogramming of the host metabolism translates in coordinated up and down regulation of genes encoding key glycolytic enzymes and glucose transporters, reminiscent of the Warburg effect as well as regulation of HIF-1 $\alpha$  (55). While the granuloma becomes necrotic, MPs packed with lipid droplets transform into foamy cells (56) which is driven by reprogramming of host lipid metabolism in response to Mtb compounds (57). Interestingly, lipid droplets formation in Mtb infected MPs is driven by IFN- $\gamma$  and requires HIF-1 $\alpha$  and its target gene *hig2* (58).

Several immunopathology studies demonstrate extensive vascularization of TB granulomas in humans (59) and mice (59, 60) provided that they are not necrotic (61). The link between hypoxia, vascularization, and development of the granuloma was recently established in the ZF infected with Mm (62). In this model, HIF-1 $\alpha$  is activated, PHD-3 expression is increased and induces production of the angiogenic factor VEGF-A, which is intimately linked to nascent granuloma formation. In human MPs infected with Mtb, a similar angiogenic signature is observed (63). Moreover, the level of VEGF-A is increased in sputum and peripheral blood of active TB patients and is proposed as a differentiating biomarker for patients progressing to active TB (64–66). Circulating angiogenic factors are markers of disease severity and are associated to the bacterial burden (67). In ZF embryos infected by Mm, CXCR4 signaling is important to initiate angiogenesis for granuloma expansion (68). Surprisingly, despite the established over-representation of neutrophils in

TB lesions, little information is available on how these cells behave in face of Mtb in the highly hypoxic and angiogenic granuloma milieu.

Neutrophils are generally seen as short-lived cells. However, the life span of neutrophils is highly increased in hypoxic milieu (69). By high consumption of oxygen during oxidative burst, neutrophils themselves contribute to generate the hypoxic milieu, which may well be the case during active TB when they invade the lung. Prolonged survival is linked to sustained expression of PHD3, *in vitro* and *in vivo*, in response to hypoxia and inflammatory stimuli (70). Interestingly, PHD3 is strongly induced in lungs of Mtb infected mice (55). HIF-2 $\alpha$  is the most expressed in neutrophils, in contrast to MPs where HIF-1 $\alpha$  is the most active. HIF-2 $\alpha$  deficiency increases neutrophil apoptosis (71). MIP-1 is also identified as a novel hypoxia stimulated granulocyte survival factor (72).

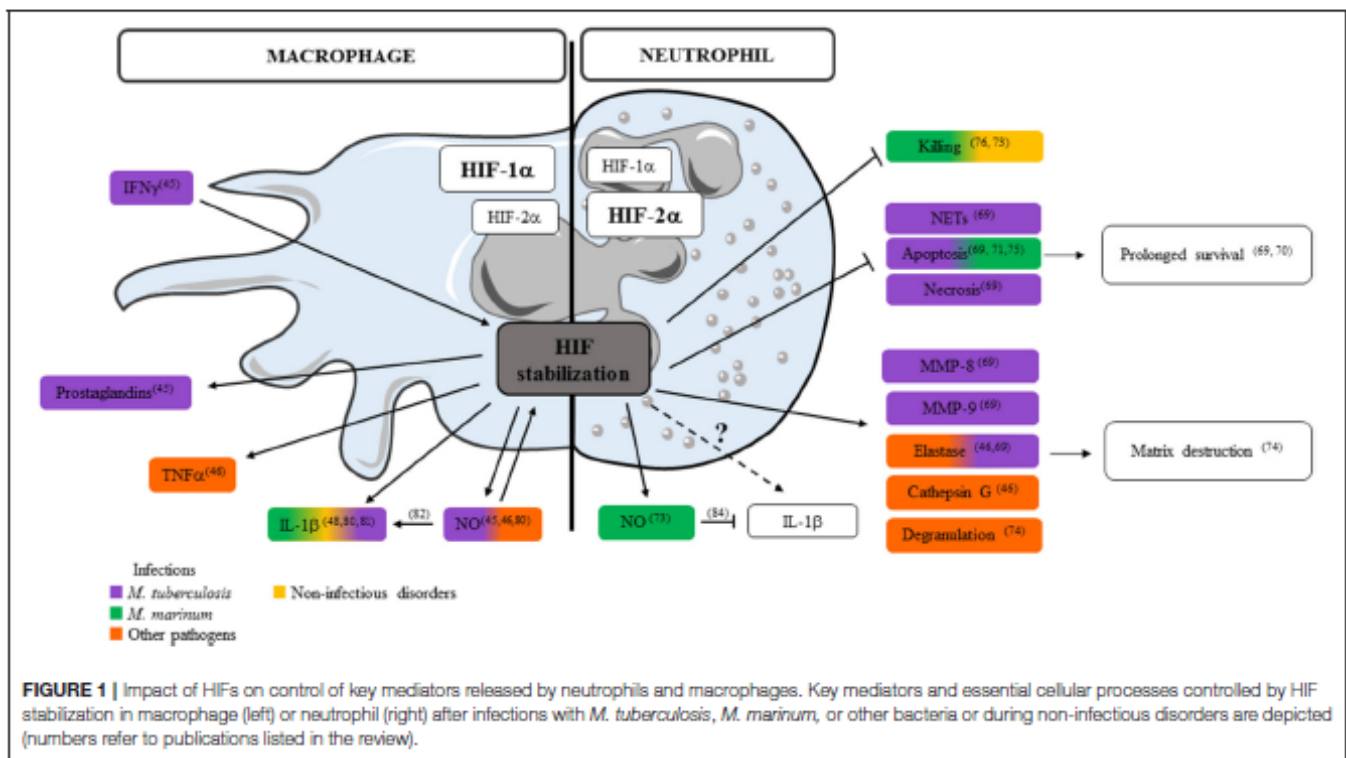
In the ZF model infected with Mm, neutrophil-specific HIF-1 $\alpha$  stabilization decreases bacterial burden via a NO-dependent mechanism. On the contrary, despite also being upregulated, HIF-2 $\alpha$  has a negative impact on bacterial burden emphasizing opposite roles of different HIF factors (73). Therefore, it is possible that the hypoxic environment of the TB granuloma that favors extended life-span for neutrophils allows them to actively shape granuloma evolution. On one hand, this may help bacilli control as well as resolution of inflammation, since neutrophils actively participate to MP efferocytosis and the release of lipids such as LXA4. On the other hand, hypoxia increases neutrophil degranulation and confers extended activity to damage lung tissues in a PI3K dependent pathway (74). Hypoxia-induced decrease of neutrophil apoptosis

induces a delay in resolution of inflammation by maintaining active neutrophils in the inflamed tissue (75). Moreover, hypoxia impairs the ability of neutrophils to kill certain bacteria (76).

HIF-1 $\alpha$  is a major player in an another chronic infection caused by the intracellular parasite Leishmania. HIF-1 $\alpha$  crucially enhances immunosuppressive functions of MDSCs and decreases leishmanicidal capacity of myeloid cells (77). Even though to our knowledge no study has tackled the link between HIF-1 $\alpha$  and MDSCs in TB, a similar important role could be discovered. Also, since hypoxia and angiogenesis are intimately linked to the granuloma development, another interesting perspective is the possible role of angiogenic neutrophils (40, 42) in the process.

### POSSIBLE INFLUENCE OF THE HYPOXIC Mtb GRANULOMA ON KEY NEUTROPHIL-RELEASED MEDIATORS

Neutrophils contribute both pro- and anti-inflammatory factors in TB (78, 79). Information on how HIF-1 $\alpha$  stabilization in hypoxic environment influences the secretion of critical immune mediators by neutrophils is limited to granule proteases, antimicrobial peptides and TNF (46). Literature on the impact of HIF-1 $\alpha$  stabilization on MP-released mediators is more extensive and we consider it as a source of inspiration illustrating the potential role of hypoxia on neutrophil-released mediators (Figure 1). In the following paragraph, we focus on how hypoxia may regulate release by neutrophils of the key mediators





**TABLE 1** | Impact of neutrophils and close relatives in cancer and TB.

	Tumor formation and evolution	Early Mtb infection and granuloma
Prognosis/Pathophysiology	<ul style="list-style-type: none"> <li>Clinical evidence (neutrophil to lymphocyte ratio) mostly links neutrophils to cancer progression. Poor prognosis.</li> <li>Different granulocytic populations described with various functions. Anti-tumor activity of High Density Neutrophils (early stage tumor). Accumulation of immature neutrophils associated to cancer progression (Gr MDSCs or Low Density Neutrophils). Promote angiogenesis, tumor progression, and metastases (95)</li> <li>In many cancers, different granulocyte subpopulations are described (96). Better definition is needed</li> </ul>	<ul style="list-style-type: none"> <li>Early phase of infection: neutrophils contribute to innate resistance (11, 97) and granuloma formation (7, 98, 99)</li> <li>Late phase of infection, active TB: established role of neutrophils to severe forms in preclinical models (8) and in humans (9)</li> <li>Gr-MDSCs accumulate during early phase Mtb infection and active TB, in blood and lung in humans (31)</li> <li>MDSCs may represent permissive reservoir for Mtb (34) and their accumulation associates with severe TB in mice (35)</li> </ul>
Hypoxia and angiogenesis	<ul style="list-style-type: none"> <li>HIF-2<math>\alpha</math>, selectively modulates neutrophil recruitment (100)</li> <li>Neutrophil recruitment to early-stage tumors is linked to hypoxia (101)</li> <li>Induction of angiogenic neutrophils in hypoxic conditions</li> </ul>	<ul style="list-style-type: none"> <li>Hypoxia augments neutrophil degranulation and confers enhanced potential for damage to respiratory airway epithelial cells (69)</li> <li>Hif-1<math>\alpha</math> increases NO production by neutrophils in early stage of Mm infection and is involved in control of bacterial growth (73)</li> <li>Granuloma formation in the ZF model coincides with angiogenesis and local hypoxia (62)</li> </ul>
Modulation of T cell response	<ul style="list-style-type: none"> <li>MDSCs are major players in tumor-mediated immunosuppression</li> <li>Neutrophils in solid tumors are potent producers of Arg-1 and could contribute to local immune suppression (102, 103)</li> </ul>	<ul style="list-style-type: none"> <li>MDSCs are present in lungs (3) but their role in development and evolution of granuloma remains unclear</li> <li>Arg-1 is associated to severe TB in mouse models (104, 105) and is detected in necrotizing granulomas in humans (106). Pathway documented in MPs, however, deciphering neutrophil contribution to Arg-1 production would be of interest.</li> </ul>
Tissue Remodeling	<ul style="list-style-type: none"> <li>MMP-9 delivered by tumor-recruited neutrophils is associated to tumor angiogenesis and dissemination (107)</li> <li>Angiogenic neutrophils contribute to tumor growth and metastasis (108)</li> <li>Neutrophils through COX-2-mediated PGE2 synthesis and elastase promote tumor cell proliferation (109)</li> </ul>	<ul style="list-style-type: none"> <li>MMPs are involved in early granuloma formation and participate to tissue destruction during late phase (110, 111)</li> <li>Pathogenic mycobacteria (Mm or Mtb) exploit the formation of new blood vessels to disseminate via MPs (62, 63). Neutrophils are migrating cells (112), their contribution to Mtb dissemination is not documented yet.</li> </ul>

in Mtb virulence: ROS, NO, IL-1 $\beta$ , and type I IFN. Some of these mediators may play different roles in humans and animal models and data should sometimes be interpreted with caution. In Mtb infected MPs, HIF-1 $\alpha$  is stabilized by IFN- $\gamma$  and regulates the production of prostaglandins and NO (45). In mice, NO not only acts as an antimicrobial agent and inflammatory mediator but further amplifies myeloid cell bactericidal activity via HIF-1 $\alpha$  stabilization. NO modulates the MP response to Mtb through activation of HIF-1 $\alpha$  and repression of NF-kB (80). HIF-1 $\alpha$  and NO are intrinsically linked: they positively regulate each other, but display distinct roles in the regulation of inflammation. Among the mediators regulated in opposite directions, neutrophil-attracting chemokines, IL-1 $\alpha$  and IL-1 $\beta$ , are all down regulated in HIF-1 $\alpha$ <sup>-/-</sup> and upregulated in Nos2<sup>-/-</sup> Mtb infected and IFN- $\gamma$  activated BMDMs (80). In the hypoxic granuloma, NO produced by IFN $\gamma$ -activated MPs restricts neutrophil recruitment to avoid destructive inflammation (80). In Mm infected ZF, HIF-1 $\alpha$  stabilization induced IL-1 $\beta$  production by MPs and increased neutrophil NO production that is protective against infection (81). In Mm infected NADPH oxidase 2-deficient mice (Ncf1<sup>-/-</sup>) mice, ROS-deficiency decreases IL-1 $\beta$  production by MPs but induces early and extensive neutrophilic inflammation, with high elastase activity and IL-1 $\beta$  production (82). This also reveals a novel role for ROS in the early neutrophilic granulomatous inflammation

and the importance of neutrophil-driven IL-1 $\beta$  production during mycobacterial infection. In addition to MPs, neutrophils also produce ROS and NOS. Neutrophils are able to discriminate pathogens by differential production and localization of ROS, and tune their own recruitment and distribution to exquisitely tailor the anti-microbial response (83). HIF-1 $\alpha$  stabilization in neutrophils induces NO production after infection by Mm (73). NOS and ROS production also influences the secretion of cytokines. NO inhibits NLRP3-dependent IL-1 responses (84). IL-1 $\beta$  signaling is also important for ROS production as Mtb-infected newly recruited neutrophils lacking IL-1R fail to produce ROS, resulting in compromised pathogen control (85). HIF-1 $\alpha$  stabilization clearly influences ROS/NOS and IL-1 $\beta$  production by MPs and neutrophils, both factors are important during mycobacterial infection, but their regulation seems different in the two cell types (73, 80–82, 84, 85) (**Figure 1**). In human neutrophils stimulated with Mtb, hypoxia up-regulates secretion of MMP-8, MMP-9 and neutrophil elastase that are involved in matrix destruction. Hypoxia inhibits NETs formation and both neutrophil apoptosis and necrosis after direct stimulation by Mtb (69).

Type I IFN is a major cytokine in TB pathophysiology. Overproduction of type I IFN (IFN-I) is linked to exacerbated TB in both mouse models and humans. IFN-I triggers immunopathology by increasing the recruitment of

inflammatory monocytes and neutrophils to the lung (86). Secretion of IFN-I by MPs and its effect on neutrophils is well-documented (87–89). In MPs, Mtb triggers IFN-I secretion through bacterial DNA release in the cytosol. However, strains display variable ability to activate the IFN-I pathway depending on their effective triggering of mitochondrial stress (87). Host-protective cytokines such as TNF, IL-12, and IL-1 $\beta$  are inhibited by exogenous IFN-I, via production of immunosuppressive IL-10 (88). By contrast, IL-1 $\beta$  suppresses IFN-I through eicosanoid prostaglandin E2 (90). In the inflammatory environment in mouse tumor models, IFN-I shifts neutrophils to antimetastatic phenotype (89). Therefore, IFN-I has multiple and crucial effects on neutrophils, but so far studies on IFN signaling in neutrophils in hypoxic environment are still scarce. As hypoxia leads to accumulation of cytosolic DNA via mitochondrial or nuclear DNA damage, it could favor activation of the cGAS/STING/IRF3 pathway (91). The convergence between hypoxia and IFN-I signaling is suggested by Karuppagounder et al. who identified the effect of Tilorone, a small molecule inducing IFN-I which also triggered hypoxic response in brain cells (91). Another study claims that IFN-I promotes tumorigenic properties through up-regulation of HIF functions in different cancer cell lines (92). Direct IFN-I secretion by neutrophils is proposed, where Sox2 could act as a sequence-specific DNA sensor in neutrophils during microbial infection (93). However, it is unclear if neutrophils can sense DNA via the cGAS/STING pathway (94).

Thus, even though the impact of hypoxic environment encountered in the TB granuloma on the IFN-I pathway is not documented yet, this issue could be of great interest to better understand TB pathophysiology and propose new therapies.

## NEUTROPHILS IN TB: MANY OPEN QUESTIONS

Although it is now consensus that during active TB, neutrophils are the main villains responsible for lung destruction, we—and others (3, 11)—advocate that this narrow vision is revisited. “Neutrophils” encompass different subsets with various functions that remain poorly defined. They come to infectious foci in waves of different magnitude. A better definition of neutrophil subsets, their coordinated dynamics of recruitment to the lung and their associated functions is needed. Neutrophils crosstalk with other cells and secrete a vast number of mediators thus taking full part to the regulation of the immune response against Mtb. They respond to signals sent by their environment, including hypoxia in inflamed tissues. In the hypoxic TB granuloma, light has been recently shed on the fate and behavior of MPs, under the control of the master regulator HIF-1 $\alpha$ . However, there is currently scarce information on the fate and behavior of neutrophils in a similar context. How do neutrophils respond to hypoxia in the TB context? How do neutrophils contribute to the shaping of the granuloma? In the future, models allowing development of the hypoxic TB granuloma should be favored. A better definition

of mediators released by neutrophils in the hypoxic context of the granuloma is expected. As neutrophils are key players in the game, we believe that these questions need to be solved in order to propose new interventions to fight against TB.

## PERSPECTIVES FOR INNOVATIVE THERAPEUTICS AGAINST TB

In the era of increasing multidrug resistance of Mtb strains, HDTs sometimes represent the last hope for patients. As the hallmark of destructive inflammation, neutrophils are often considered as potential targets. Inhibiting necrotic neutrophil death could restore Mtb growth control (24). Removing neutrophils by drugs or immunotherapeutic interventions could also alleviate lung tissue destruction.

Recent studies in the TB field shed some light on parallels that could be drawn between the TB granuloma and solid tumors (Table 1), especially regarding the role of neutrophils. Since HDTs are more advanced in the field of cancer than they are in TB, we propose that some tracks well-developed in cancer therapy are explored to advance the field of HDTs for TB patients. Among promising avenues, we propose that metabolic changes occurring in TB granuloma are being considered (113). Modulation of the HIF pathways (114) deserves attention as it could dampen excessive protease secretion (69). PHD3 and HIF-2 $\alpha$  that operate in neutrophils under inflammatory or hypoxic conditions (70, 71) represent more attractive targets than largely distributed HIF-1 $\alpha$ . In cancer, another active field in the clinics is blocking angiogenesis since this pathway is key to tumor development. Angiogenesis appeared more recently as key to the development of the TB granuloma and it would be interesting to determine whether modulating angiogenesis could bring some benefit to TB patients. Along this line, we believe that recently described angiogenic neutrophils should also be investigated in TB.

TB still kills 1.7 million people each year and active TB patients continuously spread bacilli that represent threat to human kind. Development of adjunct HDTs is a promising avenue to boost current drug regimen directed against Mtb (115). We believe that addressing the questions that we raised in this review about neutrophils in TB could greatly help in the quest for innovative HDTs.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Neutrophils Encompass a Regulatory Subset Suppressing T Cells in Apparently Healthy Cattle and Mice

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Neutrophils that reside in the bone marrow are swiftly recruited from circulating blood to fight infections. For a long time, these first line defenders were considered as microbe killers. However their role is far more complex as cross talk with T cells or dendritic cells have been described for human or mouse neutrophils. In cattle, these new roles are not documented yet. We identified a new subset of regulatory neutrophils that is present in the mouse bone marrow or circulate in cattle blood under steady state conditions. These regulatory neutrophils that display MHC-II on the surface are morphologically indistinguishable from classical MHC-II<sup>neg</sup> neutrophils. However MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils display distinct transcriptomic profiles. While MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils display similar bacterial phagocytosis or killing activity, MHC-II<sup>pos</sup> only are able to suppress T cell proliferation under contact-dependent mechanisms. Regulatory neutrophils are highly enriched in lymphoid organs as compared to their MHC-II<sup>neg</sup> counterparts and in the mouse they express PDL-1, an immune checkpoint involved in T-cell blockade. Our results emphasize neutrophils as true partners of the adaptive immune response, including in domestic species. They open the way for discovery of new biomarkers and therapeutic interventions to better control cattle diseases.

**Keywords:** neutrophil, regulation, cattle, T-cell, suppression, subset

## INTRODUCTION

Neutrophils are major partners of the innate immune system and are considered the first line of defense against microbes (1). They originate from the bone marrow (BM) and circulate in large numbers in the blood from where they are swiftly recruited to sites of inflammation or infection to fight danger. They are functionally equipped to rapidly phagocytose invading microbes *via* a variety of receptors (2) and rapidly deliver large amounts of reactive oxygen species (ROS) into phagocytic vacuoles to kill microbes (3). If not tightly controlled these dangerous weapons lead to tissue damage and neutrophils are often a signature of uncontrolled inflammation or infection.

Neutrophils are the dominant cell population circulating in blood in a wide range of animal species and humans (4). However, in cattle or mouse blood they are less numerous than lymphocytes (5, 6). Compared to human or mouse neutrophils, bovine neutrophils present peculiarities such as unique receptors or the lack of N-formylated chemotactic peptide receptor (5). Neutrophil counts are an important signature of cattle condition. In lactating cows, they

represent the most part of an abnormal somatic cell count in milk from mastitic cows. Indeed, their swift recruitment into the mammary gland is of critical importance in the fight against invasive pathogens (7). For a long time, neutrophils were thought to only travel from their birth place, the BM, to blood and tissues in response to inflammatory signals or infections. However, this has recently been revisited by a number of authors who have identified neutrophils residing in spleen (8, 9), lymph nodes (10), or lung vasculature (11). These new locations and features of resident neutrophils have been discovered in mice and humans but are not well documented in other animal species, including cattle, under normal conditions.

Long considered as “suicide bombers,” neutrophils were recently upgraded as global players of the immune response along with the discovery of new functions (12). They fully participate in shaping adaptive immunity by suppressing T cell proliferation and activity (13, 14) or by promoting IgM and IgG production by splenic B cells (9). This broad range of phenotypes and functions was recently linked to discrete populations of neutrophils characterized by unbiased single cell analyses (15). Like macrophages and dendritic cells, neutrophils are now recognized as plastic cells able to respond and adapt to their environment. Again, although neutrophil diversity and plasticity are now well documented for mouse and human neutrophils, they remain unknown for domestic species including cattle.

Here we conducted a parallel thorough characterization of neutrophils present in mouse BM or circulating in cattle blood at steady state. We define this term as animals without any apparent sign of disease or infection. In other words, animals where the immune system is at homeostasis. We discovered a new population of regulatory neutrophils that displayed suppressive activity on T-cells, at steady state.

## MATERIALS AND METHODS

### Animal Protocols and Sampling

Experimental protocols complied with French law (Décret: 2001–464 29/05/01) and European directive 2010/63/UE for the care and use of laboratory animals and were carried out under Authorization for Experimentation on Laboratory Animals Number D-37-175-3 (Animal facility UE-PFIE, INRA Centre Val de Loire for mice) and E 37-175-2 (UE-PAO, INRAE Centre Val de Loire for bovine). Animal protocols were approved by the “Val de Loire” Ethics Committee for Animal Experimentation and were registered to the French National Committee for Animal Experimentation under N°2016091610026164.V3 (mice) or N°2016040410531486 (cattle). Cattle remained in their environment (UE-PAO) and no animal was sacrificed for this work. Six- to eight-week-old C57BL/6 mice and OT-II transgenic mice for the OVA MHC class II complex-specific TCR (16) on a C57BL/6 genetic background were bred at the resident PFIE animal facility before use.

Bovine blood was collected from Holstein Friesian cows at the jugular vein into vacutainer K2 EDTA tubes. Broncho-tracheal lymph nodes, spleen biopsy, and sternum BM were collected *post-mortem* at a commercial abattoir from Blonde d'Aquitaine,

Limousine, Charolaise and Highlands cows. Mice were euthanized by CO<sub>2</sub> inhalation. Blood (from heart), spleen, inguinal lymph nodes, and femurs were collected.

### Preparation of Cells

Tubes containing blood were centrifuged at 1000xg for 10 min at 20°C before removal of the plasma layer and buffy coat. In indicated experiments, buffy coat was used in proliferation assay (see below). Red blood lysis buffer (Sigma 94 R7757) (4 vol/1 vol of blood) was added for 5 min at room temperature to lyse red blood cells. Cells were washed twice in D-PBS with 2mM EDTA. Bovine blood neutrophils were labeled with primary anti-G1 and anti-MHC-II Ab, followed by secondary fluorescent-conjugated Ab before purification by FACS (see next paragraph for labeling protocol). Mouse neutrophils were generally purified from the BM by positive magnetic selection with anti-Ly-6G PE-conjugated Ab (IA8; BD Biosciences) and anti-PE microbeads (Miltenyi Biotec) as described previously (17) except for cell sorting, where mouse neutrophils were enriched from BM by positive magnetic selection with anti-CD11b microbeads (Miltenyi Biotec) before labeling with antibodies. Bovine BM cells were collected *post-mortem* at local commercial abattoirs, by scratching an open rib (directly on the carcass) with a spatula, cells were diluted in PBS. Spleen biopsies, the broncho-tracheal lymph nodes (LN) and BM cells were transported at 4°C to our laboratory. Bovine and murine LN and spleen biopsies were disrupted mechanically and all cells were filtered through 100µm nylon cell strainer (BD Falcon), and washed twice. Cells were suspended in RPMI-1640 supplemented with 2mM L-glutamine, 10mM HEPES, and 1mg/ml of BSA with extremely low endotoxin level ( $\leq 1.0$  EU/mg) (hereafter referred to as RPMI complete medium). Cell counts were determined after staining with Türk solution and numerated with a Malassez's chamber.

### Flow Cytometry

Bovine cells were suspended in PBS with 10% of horse serum (Gibco), 2mM EDTA, and labeled for 30 min with primary antibodies (see **Supplementary Table S1**). Mouse cells were suspended in PBS 2mM EDTA. After saturation with anti-CD32/CD16, murine cells were incubated 30min with fluorescent mAb. After washes in D-PBS (300xg, 10 min, 4°C), both murine and bovine cells were labeled 30min with the corresponding fluorescent-conjugated secondary antibodies. Cells were washed and fixed with BD cell fix diluted four times in PBS. Data were acquired with a LSR Fortessa™ X-20 Flow cytometer (Becton Dickinson) and results analyzed with Kaluza software (Beckman Coulter).

For neutrophil subsets purification, cell concentrations were adjusted to  $10^7$  cells/ml and sorted with a MoFlo Astrios<sup>BQ</sup> high speed cell sorter (Beckman Coulter) according to our previously published protocol (18). Sorted cells were spread on microscope slides (Superfrost, Thermo) by cytocentrifugation (3 min, 700xg) and stained with May-Grünwald and Giemsa with the RAL 555 kit (RAL Diagnostics).

### Neutrophils Functional Assays

Sorted neutrophils viability was evaluated after 1 h stimulation with 100ng/ml of LPS (*Escherichia coli* 0111:B4, Sigma). Neutrophils



were then incubated 15 min at room temperature with anti-annexin V antibodies in binding buffer (BD Bioscience). Cells were washed and incubated 15 min at room temperature with streptavidin-APC-Cy7 (BD Bioscience). Cells were washed and incubated 5 min with 20 µg/ml of propidium iodide (BD Bioscience) and then directly analyzed with LSR Fortessa X-20 flow cytometer. Phagocytosis was measured using pHrodo™ Red *E. coli* BioParticles® Conjugated (MolecularProbes®) following the manufacturer's instructions. Briefly, purified neutrophils were first incubated in a 96 wells microplate for 30 min at 37°C in RPMI complete medium with or without 2 µg/well of cytochalasin D (Sigma), and then for 1 h with 20 µg/wells of pHrodo *E. coli* BioParticles. Fluorescence was directly measured with the LSR Fortessa™ X-20 Flow cytometer. ROS produced by neutrophils were quantified using the CellROX® Orange Flow Cytometry Assay Kits (MolecularProbes®, C10493) following the manufacturer's instructions. Briefly, purified neutrophils were first incubated for 1 h at 37°C in RPMI complete medium with or without 400 µM of TBHP in a 96-wells black microplate and then for 30 min with 100 nM CellROX®. Fluorescence was measured with the LSR Fortessa™ X-20 Flow Cytometer. To test the bacterial killing capacity of neutrophils, we used the *Escherichia coli* P4 strain, isolated from a clinical case of bovine mastitis which causes severe infections in mice (19). *Escherichia coli* P4 bacteria were grown in 10 ml BHI medium overnight at 37°C without agitation. Bacteria were then diluted in BHI medium (1 vol/100 vol) and incubated for 6 h at 37°C without agitation. Bacterial concentration was determined by the optical density at 600 nm and adjusted at 4.10<sup>5</sup> CFU/ml in RPMI complete medium. Purified neutrophils were infected at a MOI of 0.2 in RPMI complete medium in 1.5 ml Eppendorf tubes for 90 min at 37°C under agitation on a Rotator SB3 (Stuart equipment). Bacteria in RPMI complete medium without neutrophils were used as the reference to assess killing. Bacterial dilutions (in PBS 0.25% SDS) were plated on TSA agar plates and incubated for 16 h at 37°C before enumeration of CFUs.

## RNA Extraction and Gene Expression Analysis

Total RNAs were extracted from cell-sorter purified cells using NucleoSpin RNA kit with a DNase treatment (Macherey Nagel) and reverse transcribed with iScript™ Reverse Transcriptase mix (Bio-Rad) according to the manufacturer's instructions. Primers (Eurogentec) are listed in **Supplementary Table S2**. Primers validation was performed on a serial diluted pool of cDNA (a mix of cDNA from spleen, lung, LN, blood, and BM cells for both species) with a LightCycler® 480 Real-Time PCR System (Roche). Gene expression was then assessed with the Biomark HD (Fluidigm) in a 48x48 wells plate, according to the manufacturer's instructions. The annealing temperature was 60 and 62°C for bovine and mouse samples respectively. Data were analyzed with Fluidigm Real-Time PCR software to determine the cycle threshold (Ct) values. Messenger RNA (mRNA) expression was normalized to the mean expression of three housekeeping genes for each animal species to obtain the ΔCt value. Principal component analysis (PCA) and hierarchical clustering were performed with ΔCt values in R studio

(Version 1.3.959, © 2009-2020 RStudio, PBC), using respectively the FactoMineR and pheatmap packages. Ward's minimum variance method was applied for clustering, with dissimilarities squared before clustering (ward.D2). ΔCt values were centered to the median for clustering.

## Measure of T-Cell Suppressive Activity of Neutrophils

For the mouse, splenocytes from OT-II mice were collected, homogenized to single-cell suspensions through nylon screens and resuspended in RPMI medium (Gibco) supplemented with 10% decomplexed fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 U penicillin, and 100 µg/ml streptomycin (Gibco). 10<sup>5</sup> cells/well were distributed in a 96-wells round bottom plate (BD Falcon). OT-II splenocytes proliferation was induced by addition of 2 µg/ml of the OVA peptide 323-339 (PolyPeptide Group). As indicated, purified neutrophils were added to the culture at a ratio of 1 neutrophil:10 splenocytes in a final volume of 200 µl. Wells without neutrophils were used as reference for maximal proliferation.

As indicated, neutrophils were separated from splenocytes by placing them in a HTS Transwell-96 permeable device with 0.4 µm pore and polycarbonate membrane and adapted receiver plate (Corning, reference CLS3381). To test the role of MHC-II and CD11b molecules in the suppression mechanism, neutrophils were also treated 1 h before incubation with splenocytes with 15 µg/ml anti-CD11b mAb (clone M1/70) or rat IgG2b<sub>k</sub> as isotype control; anti MHC-II mAb (clone 2G9) or rat IgG2a<sub>k</sub> as isotype control. Plates were incubated at 37°C with 5% CO<sub>2</sub>. Cell proliferation was quantified after 3 days of culture using CyQUANT Cell Proliferation Assay tests (Thermo Fisher) according to the manufacturers' instructions.

For the bovine, a mixed lymphocyte reaction assay was set up by mixing blood cells from two genetically distant cows with cow N°1 as the responding animal and cow N°2 as the stimulating animal. Briefly, blood was centrifuged at 1000xg, 15 min, 20°C, and buffy coats were collected and diluted four times in PBS. PBMCs were collected at the interface of 1.077 density Percoll gradient (GE Healthcare) after centrifugation at 400g, 15 min, 20°C, without brake. PBMCs from cow n°1 (responding) remained untreated while PBMCs from cow n°2 (stimulating) were incubated for 30 min at 38.5°C in 5% CO<sub>2</sub> with 50 µg/ml of mitomycin C from *Streptomyces caespitosus* (Sigma, M4287), to block their proliferation. After three washes, 10<sup>5</sup> stimulating cells were mixed with 10<sup>5</sup> responding cells in 96 wells plates (ratio 1:1) in a total volume of 150 µl in RPMI complete medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The negative control was responding cells alone and the reference maximal proliferation was responding and stimulating cells together at ratio 1:1. Proliferation that started at day 6 was stopped at day 9. As indicated, at day 4, 10<sup>5</sup> of purified syngenic neutrophils from the responding animal were added to the well in a final volume of 200 µl. In one experiment, neutrophils were placed in a HTS Transwell-96 permeable device as for the mouse system. To quantify proliferation, plates were centrifuged at day 6 and day 9 for 5 min at 300xg, supernatants were discarded and cells were



frozen at  $-80^{\circ}\text{C}$  before addition of reagents from the CyQUANT Cell Proliferation Assay Kit (Thermo Fisher) as indicated by the manufacturer.

## Statistical Analysis

Individual data and the median were presented in the figures. Statistical analyses were performed with Prism 6.0 software (GraphPad). Analyses were performed on data from two to six independent experiments, Mann Whitney non-parametric tests, or two-way ANOVA test were used. Represented p-values were:  $*p < 0.05$ ;  $**p < 0.01$ , and  $***p < 0.001$ .

## SUPPLEMENTARY INFORMATION

Supplemental information can be found with this article online. Should the reader need additional details, please email a request to corresponding authors.

## RESULTS

### Neutrophils Represent Discrete Populations in Mouse and Cattle

In cattle, neutrophils are often isolated by simple centrifugation of freshly collected blood to separate them from the buffy coat. They segregate at the bottom of the tube with red blood cells (20, 21). In mice, because of very small volumes, blood sampling is less practical. Therefore, high numbers of neutrophils are directly extracted from the BM by magnetic selection following labeling with antibody against Ly-6G which is highly expressed by mouse neutrophils (22). These methods, that lead to a fair level of purity for neutrophils, are convenient for most assays because they are quick and preserve these fragile cells. We prepared cattle and mouse neutrophils using these rapid procedures and analyzed them by flow cytometry (Figure 1). Following mouse bone-marrow cell preparation and labeling with anti-Ly-6G and magnetic separation (Figure 1A), banded-cells of apparent homogeneity were observed after May-Grünwald-Giemsa (MGG) staining (Figure 1B). However, the side scatter (SSC) and forward scatter (FSC) profile by flow cytometry displayed two discrete populations of heterogeneous size. Moreover, double labeling of these “pure” neutrophils with anti-Ly-6G and anti-CD11b distinguished CD11b<sup>hi</sup> from CD11b<sup>med</sup> cell populations (Figure 1C). In cattle, after elimination of the buffy coat containing PBMCs, cells were collected from lower 2/3 of the tube (Figure 1D) and analyzed after centrifugation and MGG staining. This revealed heterogeneity of this cell fraction with the presence of eosinophils in variable proportions (Figure 1E). After labeling with anti G1, a marker that is highly expressed on the surface of bovine neutrophils (23, 24) and anti CD11b, cells were analyzed by flow cytometry. After SSC and FSC gating on the granulocytes populations (Figure 1F), we distinguished CD11b<sup>pos</sup> G1<sup>low</sup> eosinophils (representing between 2 and 8% of the SSC x FSC granulocyte gate depending on the animal) from G1<sup>hi</sup> neutrophils. Among G1<sup>hi</sup> neutrophils CD11b labeling

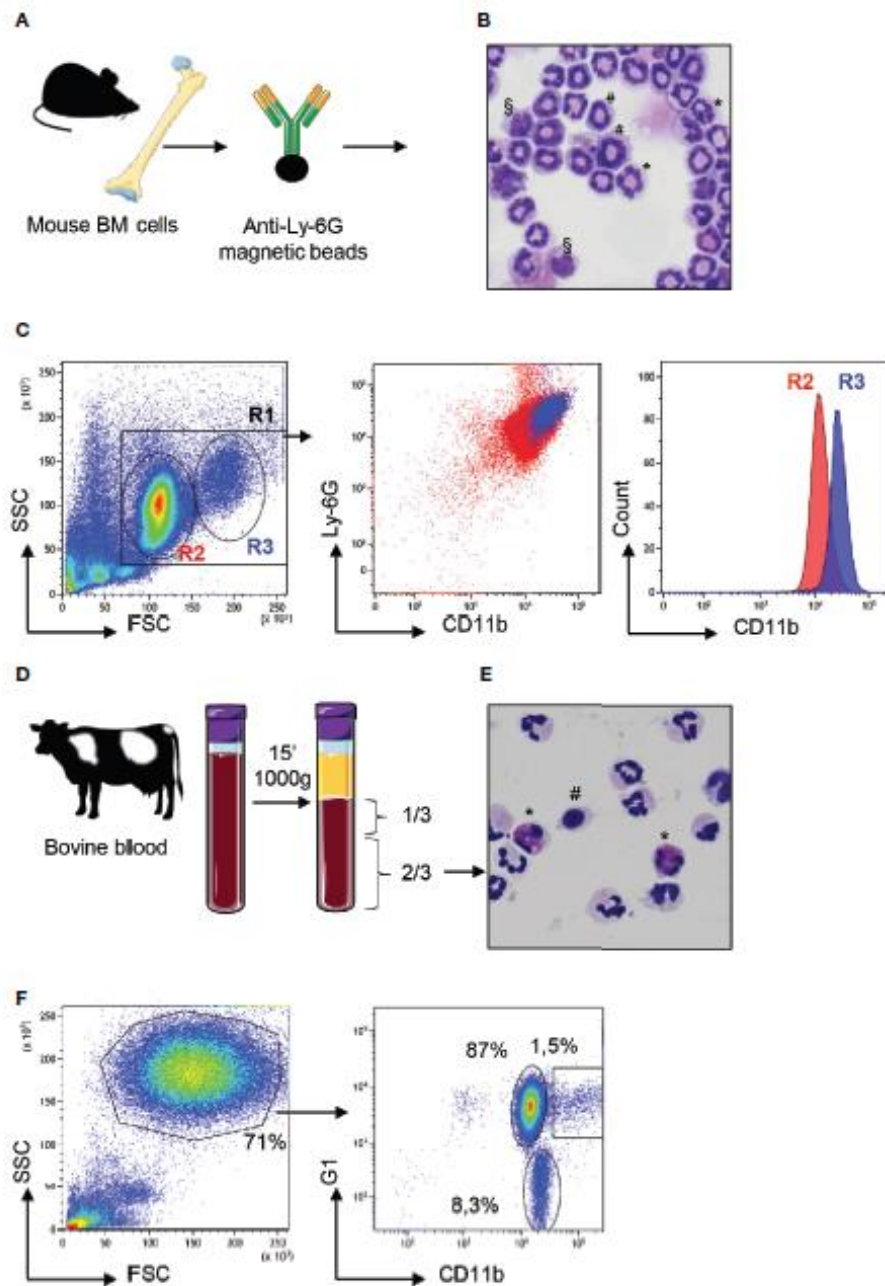
segregated two populations: a main population of CD11b<sup>med</sup> neutrophils (around 87% of the granulocyte gate) and a minor population (around 1.5%) of CD11b<sup>hi</sup> neutrophils (Figure 1F).

As we were intrigued by the presence of different neutrophil populations in mouse BM and cattle blood at steady state, we performed a more thorough characterization by flow cytometry using a panel of markers (Figure 2). In mouse, CD11b was used to gate myeloid cells (Figure 2A) and a combination of anti-Ly-6C and anti-Ly-6G allowed us to distinguish *bona-fide* neutrophils (Ly-6G<sup>hi</sup>) from monocytes (Ly-6G<sup>neg</sup>) (25). Among Ly-6C<sup>+</sup> Ly-6G<sup>hi</sup> cells, a minor subset (1.2%) of MHC-II<sup>pos</sup> Ly-6C<sup>+</sup> Ly-6G<sup>hi</sup> neutrophils was clearly distinguished from the main population (92.5%) of the MHC-II<sup>neg</sup> neutrophils. In cattle, among the neutrophils that highly expressed the G1 marker, MHC-II<sup>neg</sup> neutrophils represented the main population (93%). However, a minor population of G1<sup>hi</sup> neutrophils (around 1.5%) expressed MHC-II antigens on the surface as observed in the mouse (Figure 2A). The two MHC-II<sup>neg</sup> et MHC-II<sup>pos</sup> subsets were sorted by flow cytometry in both species. After centrifugation onto glass slides and MGG staining, they were indistinguishable under a microscope (Figure 2B). Their banded or segmented nuclei morphology, for mouse bone marrow or bovine blood neutrophils respectively, probably reflected their different maturation stage. Sorted MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils (purity >99%) were then labeled with a panel of antibodies (Figure 2C). In both bovine and mouse, in comparison with classical MHC-II<sup>neg</sup> neutrophils, the MHC-II<sup>pos</sup> subset overexpressed CD11b on the surface as well as L-selectin CD62L. The CD14 LPS coreceptor was not detected on MHC-II<sup>neg</sup> neutrophils. By contrast around 50% of MHC-II<sup>pos</sup> neutrophils displayed this receptor on their surface in both the mouse and the bovine. In mouse, we also observed that the MHC-II<sup>pos</sup> subset expressed high levels of CD44 as well as CD274 (PDL-1) the ligand for PD-1 involved in T cell exhaustion (26). Since neutrophils are fragile cells which could be exacerbated by flow cytometry sorting, we next asked if the two subsets displayed different *ex-vivo* survival times. The two subsets were incubated with LPS for 1 hour and stained with annexin V and propidium iodide (Figure 2D). The large majority of neutrophils remained alive after this treatment and no significant difference was observed between classical MHC-II<sup>neg</sup> neutrophils (97% alive in mouse or bovine) and MHC-II<sup>pos</sup> neutrophils (95% alive in mouse or bovine).

Therefore, in mouse BM or in cattle blood, two subsets of neutrophils could be distinguished at steady state by MHC-II labeling. Both subsets displayed similar survival profiles after short-term *ex-vivo* LPS stimulation.

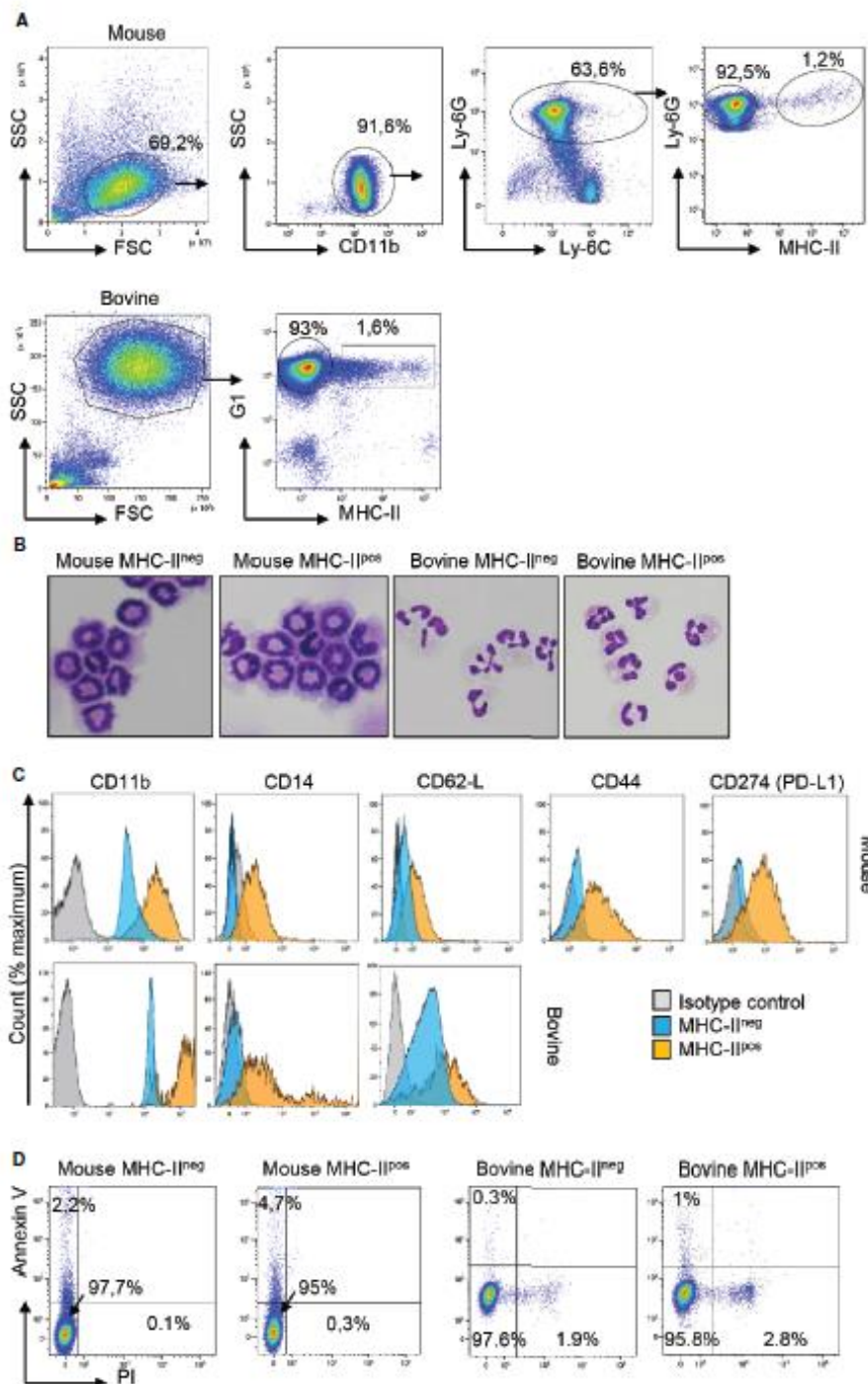
### MHC-II<sup>pos</sup> Neutrophils Are Enriched in Lymphoid Organs

Although neutrophils are mostly present in the BM reservoir and in circulating blood, recent data in the mouse model and in humans highlighted their wider distribution in tissues (27) including lymphoid organs. We next examined the distribution of MHC-II<sup>neg</sup> versus MHC-II<sup>pos</sup> neutrophils in different compartments in both mouse and cattle. Cells were extracted



**FIGURE 1 |** Neutrophils from mouse bone marrow or cattle blood display heterogeneous profiles. **(A)** Bone marrow (BM) cells from C57BL/6 adult mice were magnetically purified with anti-Ly6G beads. **(B)** These cells stained with May Grünwald Giemsa after cytoentrifugation on glass slides displayed heterogeneous profiles with segmented nuclei (\*), banded nuclei (#) as well as nuclei with a mononuclear kidney shape (S). **(C)** After anti-Ly-6G magnetic purification, mouse cells were analyzed by flow cytometry using the LSR Fortessa™ X-20 apparatus and in the “granulocytes” R1 gate, a bimodal distribution of  $FSC^{int}$  (R2) and  $FSC^{high}$  (R3) cells was observed on a linear scale (first panel). Labeling with anti-Ly-6-G and anti-CD11b confirmed this heterogeneity after dot plot analysis (middle panel) with bimodal distribution of  $CD11b^{int}$  (R2) and  $CD11b^{hi}$  (R3) cells as displayed on the histogram analysis (right panel). **(D)** Blood from Hostein Friesian cows was centrifuged and the lower 2/3 of the tube enriched in neutrophils were collected. **(E)** These cells, as observed after cytoentrifugation and staining by May Grünwald Giemsa, were mainly neutrophils, characterized by their polylobed nucleus but monocytes (#) and eosinophils (\*) were also observed in various proportions depending on the animal. **(F)** These cells were analyzed by flow cytometry and FSC x SSC dot plot showed granulocytes in a gate that represented 71% of analyzed cells (left panel). After labeling with anti-G1 and anti-CD11b, dot-plot analysis of the “granulocytes” gate (right panel) revealed three subsets: eosinophils that were negative for the G1 marker (around 8% of granulocytes) and two subsets of  $G1^{int}$  neutrophils that were  $CD11b^{int}$  (87%) or  $CD11b^{hi}$  (1.5%). The most representative animal is shown ( $n=4$  for mouse,  $n=6$  for bovine), although in cattle the proportion of eosinophils and  $CD11b^{hi}$  neutrophils circulating in blood varied between animals.





**FIGURE 2** | Two populations of neutrophils can be distinguished on the basis of specific surface markers, including MHC-II, in the mouse, and the bovine. **(A)** Cells were prepared from mouse bone marrow (BM) or cattle blood as described in **Figure 1** and neutrophils were sorted with a MoFlo Astrios<sup>FC</sup> apparatus after labeling with eFluor viability dye 780, anti-CD11b, anti-Ly-6C, anti-Ly-6G, and anti-MHC-II in the mouse or eFluor viability dye 780, anti-G1, and anti-MHC-II in the bovine. Representative plots that show the major proportion of MHC-II<sup>neg</sup> and the minor proportion of MHC-II<sup>pos</sup> neutrophils in both species are depicted. **(B)** The two MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophil populations among total CD11b<sup>pos</sup> Ly6-G<sup>pos</sup> Ly6-C<sup>pos</sup> mouse neutrophils, or G1<sup>pos</sup> bovine neutrophils, were sorted by flow cytometry (purity >99%), cytocentrifuged and were indistinguishable after May Grünwald Giemsa staining. **(C)** Histograms represent expression of CD11b, CD14, CD62-L, for both species and CD44 and CD274 (PD-L1) for the mouse samples on the surface of sorted MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils in comparison to isotype controls. **(A–C)** Results from one representative animal are depicted ( $n=6$  for mouse,  $n=6$  for bovine). **(D)** After purification by flow cytometry, MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils from the two species were incubated with LPS for 1 h and labeled with annexin-V and propidium iodide (PI) to analyze apoptotic (annexin V<sup>+</sup>; PI<sup>-</sup>) and dead cells (annexin V<sup>+</sup>; PI<sup>+</sup>). Dot plots from one representative animal are depicted ( $n=4$  for mouse,  $n=3$  for bovine).



and prepared from BM, lymph nodes (LN, inguinal in the mouse and tracheobronchial in bovine), and spleen. For analysis of circulating neutrophils, total blood leukocytes were analyzed by flow cytometry (see **Supplementary Figure S1** for isotype controls and **Supplementary Figure S2** for gating strategy) after lysis of red cells. In the BM, neutrophils represented around 30% of total leukocytes in both species (**Figure 3A**). This was expected since BM is the principal compartment hosting neutrophils. In blood, neutrophils represented 30 or 20% of total cells in mouse and cattle, respectively. In both the mouse and the bovine LN, even though neutrophils represented less than 1% of cells at steady state, they were consistently detected. Similarly, between 2 and 3% of cells collected from mouse or bovine spleen after lysis of red blood cells were neutrophils (**Figure 3A**). In all analyzed compartments classical neutrophils as well as MHC-II<sup>Pos</sup> neutrophils were detected (**Figure 3B**). Interestingly, whereas MHC-II<sup>Pos</sup> neutrophils represented only  $2.4 \pm 0.6$  or  $4.6 \pm 1.4\%$  of total neutrophils in the mouse bone-marrow or cattle blood, respectively, they represented around 40 or 60% of total neutrophils in mouse or bovine lymph nodes, respectively. In spleen, around 20% of total mouse neutrophils and 30% of bovine were MHC-II<sup>Pos</sup> (**Figure 3B**). Therefore MHC-II<sup>Pos</sup> neutrophils were highly enriched in lymphoid organs in both species, at steady state.

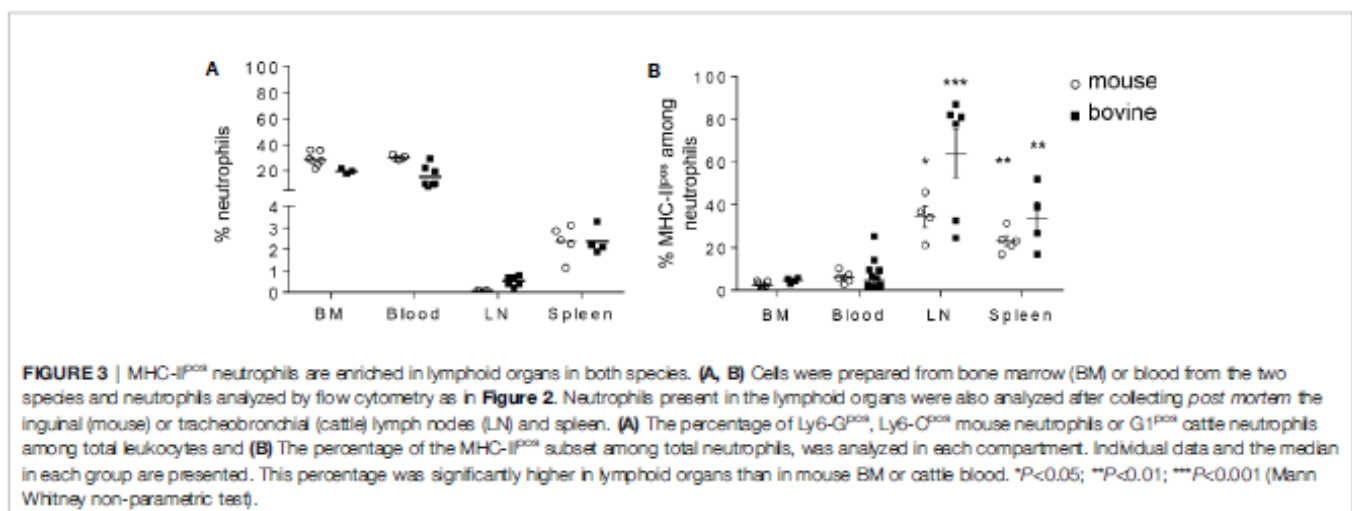
### RNA Profiling Distinguishes Classical From MHC-II<sup>Pos</sup> Neutrophils in Both the Mouse and the Bovine

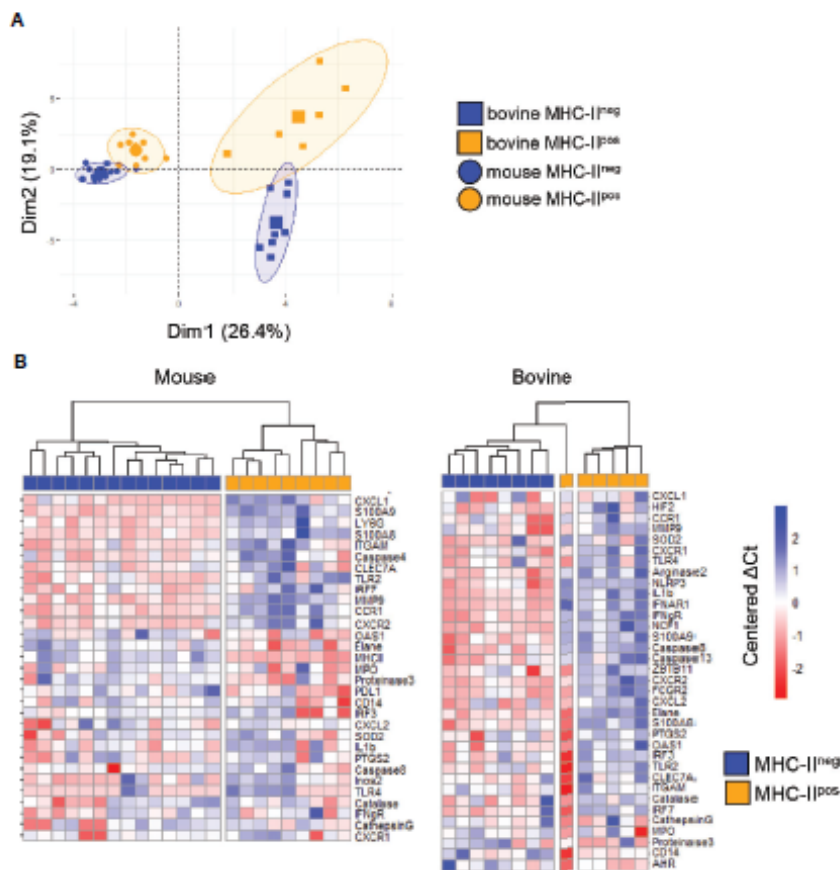
To analyze the transcriptional profile of MHC-II<sup>Pos</sup> and MHC-II<sup>Neg</sup> neutrophils, we sorted the two populations from either BM (mouse) or blood (cattle) to more than 99% purity and performed a transcriptomic profiling using 48 validated primer pairs (**Supplementary Table S1**) designed to cover a large set of neutrophil functions such as synthesis of cytokines or chemokines, enzymes stored in granules, surface receptors, as well as transcription factors. Some weakly expressed genes were removed from the panel (highlighted in **Supplementary Table**

**S1**). We then performed unsupervised principal component analysis (PCA) and observed that the different subsets were significantly discriminated (**Figure 4A**). The first axis of the PCA (explaining 26.4% of the variance) separated bovine and mouse samples, as expected. The second axis (explaining 19.1% of the variance) clearly separated the MHC-II<sup>Pos</sup> from the MHC-II<sup>Neg</sup> neutrophils in each species (**Figure 4A**), indicating that they belonged to different subsets. As expected, in the bovine, gene expression profiles were more dispersed than in the inbred mouse, due to inter-individual variability. In both species, the two neutrophil subsets were also segregated by hierarchical clustering based on  $\Delta CT$  values (**Figure 4B**). Most of the genes were significantly less expressed in MHC-II<sup>Pos</sup> neutrophils as compared to MHC-II<sup>Neg</sup>. However, expression of the following genes was significantly higher in the MHC-II<sup>Pos</sup> subset: *elane*, *proteinase3*, *PD-L1* in murine and *MHC-II*; *AHR* in bovine cells. A trend for higher expression was observed in MHC-II<sup>Pos</sup> neutrophils for *CD14* (both species) and *proteinase3* (bovine). Therefore, in both species the transcriptomic signature significantly differed between the two neutrophil subsets which suggested different biological roles.

### MHC-II<sup>Pos</sup> Neutrophils Produce Higher Levels of ROS Than MHC-II<sup>Neg</sup> but Similarly Phagocytose Bioparticles and Kill Bacteria

In order to gain further insight into the functions of the two neutrophil populations, we first compared their phagocytic ability using conjugated pHrodo™ Red *E. coli* BioParticles™ that only fluoresce once inside the phagosome or endosome of the cell (28). Neutrophils were then analyzed by flow cytometry and the percentage of phagocytosis was directly correlated with the mean fluorescence intensity (see **Supplementary Figure S3**). Phagocytosis by MHC-II<sup>Neg</sup> or MHC-II<sup>Pos</sup> neutrophils was compared in individual animals from each species. Both subsets actively phagocytosed bioparticles which was dramatically reduced by treatment with cytochalasin D (**Figure 5A**). We did not detect a significant difference in phagocytosis





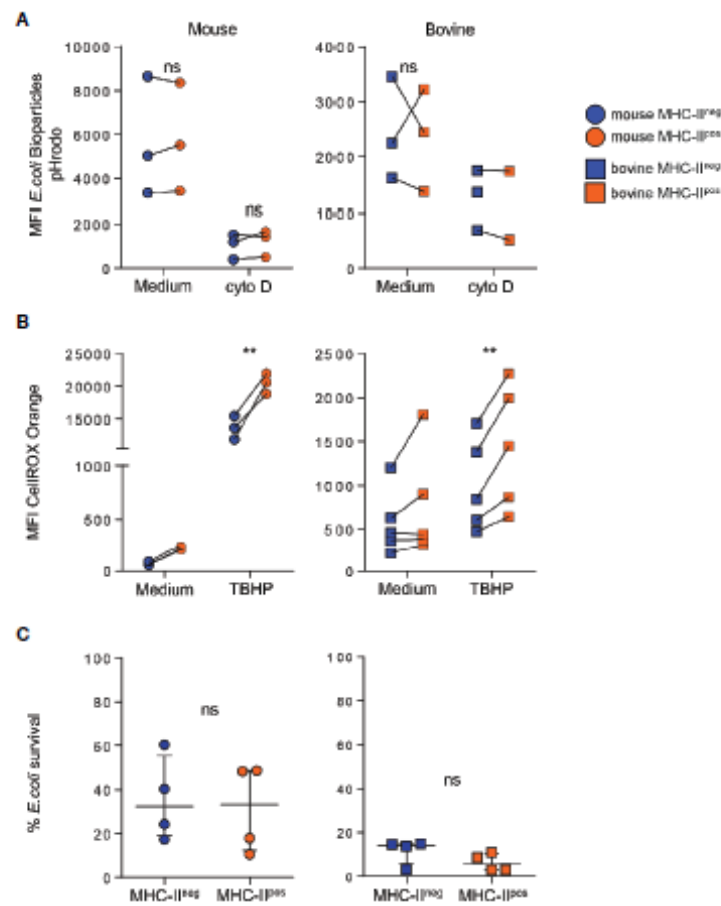
**FIGURE 4 |** MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils display distinct transcriptomic signatures. Gene expression of the two purified neutrophil subsets (more than 99% purity) from the two species was assessed by quantitative Real Time PCR using sets of primer pairs designed to cover a large range of neutrophils functions. mRNA expression was normalized to the expression of three housekeeping genes for both species to calculate the  $\Delta C_t$  values (A), principal component analysis (PCA) was performed on  $\Delta C_t$  values and the two first dimensions of the PCA plot are depicted. Inclusion in groups of sets of samples are delineated in the colored areas on the graph with a confidence level of 90% (B) Hierarchical clustering of gene expression was performed on median centered  $\Delta C_t$  values for mouse and bovine data sets, using the ward.D2 method. For each gene,  $\Delta C_t$  values were centered to the median  $\Delta C_t$  value. Higher or lower  $\Delta C_t$  expression compared to the median value were represented respectively in deep to light red or blue. Expression of a selected set of genes indicated on the figure was clearly distinct in MHC-II<sup>pos</sup> neutrophils or MHC-II<sup>neg</sup> neutrophils. Data represent individual samples (mouse: n= 14 for MHC-II<sup>neg</sup>, n=9 for MHC-II<sup>pos</sup> Bovine: n=8 for MHC-II<sup>neg</sup> n=6 for bovine MHC-II<sup>pos</sup>).

between MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils neither in the mouse nor the bovine (Figure 5A). We next measured their potential for total ROS production after incubation with the non-specific chemical inducer *tert-butyl hydroperoxide* (TBHP) using the fluorescent probe CellROX that fluoresces when oxidized (see Supplementary Figure S3). Mouse bone-marrow neutrophils incubated with medium produced very low levels of ROS whereas bovine blood neutrophils produced higher levels (Figure 5B). As expected, TBHP treatment dramatically increased ROS production by both MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils in both species. However, in both conditions and for both species, MHC-II<sup>pos</sup> neutrophils produced significantly more ROS than MHC-II<sup>neg</sup> cells (Figure 5B). We next analyzed the killing activity, under non-opsonic conditions, of the two subsets against the *Escherichia coli* P4 strain isolated from a case of bovine clinical mastitis (19). Both types of neutrophils efficiently killed *E. coli* and no significant difference was observed between MHC-II<sup>neg</sup> or MHC-II<sup>pos</sup> neutrophils (Figure 5C).

## MHC-II<sup>pos</sup> but Not MHC-II<sup>neg</sup> Neutrophils Exert Contact-Dependent Suppression of T Cells at Steady State

We wondered if surface proteins such as MHC-II (mouse and cattle) or PDL-1 (mouse) or enrichment of MHC-II<sup>pos</sup> neutrophils in lymphoid organs could be linked to regulatory functions on T cells. To address this, we set up *in vitro* assays that were either antigen specific in the mouse (Figure 6A) or polyclonal in cattle (Figure 6C). Using the OT-II transgenic mice that bear the OVA peptide 323-339—MHC class II complex-specific TCR (16) we observed strong proliferation of splenocytes when stimulated with OVA peptide for 72 hours. Taking this condition as the maximum proliferation (100%) we compared the impact of adding MHC-II<sup>neg</sup> and MHC-II<sup>pos</sup> neutrophils to the proliferating cells (ratio 10 splenocytes:1 neutrophil). The classical MHC-II<sup>neg</sup> neutrophils had no measurable effect on OT-II splenocytes proliferation (Figure





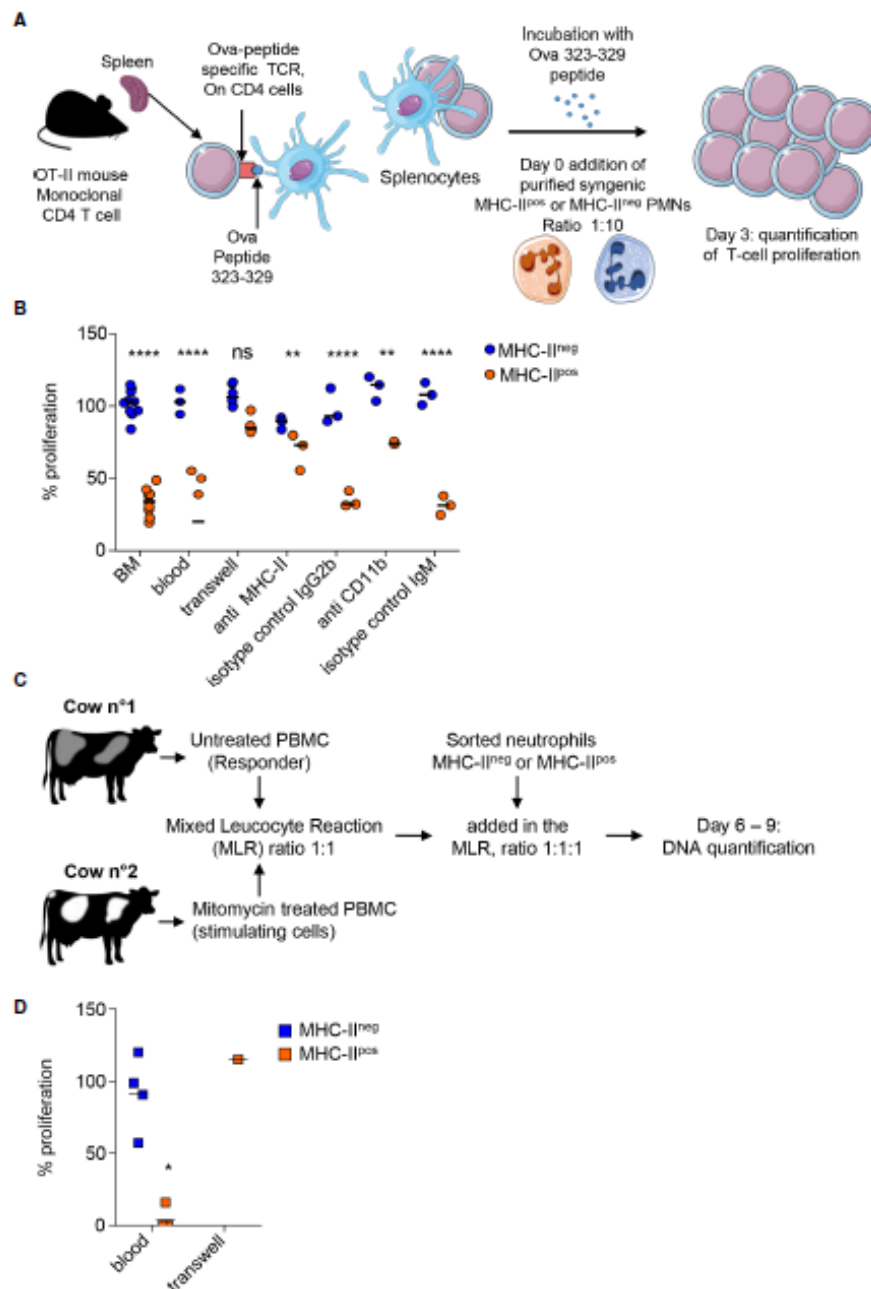
**FIGURE 5** | MHC-II<sup>pos</sup> phagocytosis bioparticles and kill *Escherichia coli* similarly to MHC-II<sup>neg</sup> neutrophils but produce higher levels of reactive oxygen species (ROS). **(A)** After purification by cell sorting from the bone marrow (BM) (mouse) or blood (bovine), phagocytosis by MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils was assessed using pHrodo *E. coli* bioparticles. Mean fluorescence intensity was directly correlated to ingested particles. Cells were treated with cytochalasin D as indicated. **(B)** Oxidative stress was measured in MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils using the CellROX Orange probe that reacts with all ROS species. Cells were activated with TBHP or incubated with medium alone and levels of ROS were measured by flow cytometry. **(A, B)** Mean fluorescence intensity in each sample is depicted and paired MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> samples were analyzed for each animal. **(C)** Purified MHC-II<sup>pos</sup> and MHC-II<sup>neg</sup> neutrophils were infected with the *E. coli* P4 strain and bacterial survival was calculated by determining the ratio of bacteria incubated alone or in presence of neutrophils. Data represent n=4 independent experiments with neutrophils pooled from independent lots of 10 mice, or prepared from blood of n=4 independent cows. **(A–C)** \*\*P<0.01; (2way ANOVA). ns, non significant.

6B). By contrast, addition of MHC-II<sup>pos</sup> neutrophils purified either from mouse BM or blood, decreased the capacity of CD4 OT-II cells to proliferate by  $66 \pm 3\%$  (Figure 6B). The suppressive activity of mouse MHC-II<sup>pos</sup> neutrophils depended on contact with the proliferating T-cells as no effect was observed when cells were separated by a Transwell device (Figure 6B). We then used blocking antibodies in order to define if MHC-II or CD11b, that were used to distinguish the two subsets as both are highly expressed on the surface of MHC-II<sup>pos</sup> neutrophils, were involved in this suppressive activity. Both anti-MHC-II and anti-CD11b partially relieved suppression by MHC-II<sup>pos</sup> neutrophils that reached  $70 \pm 7$  and  $75 \pm 1\%$  of total proliferation respectively (Figure 6B). This indicated that both molecules were involved in the suppression mechanism although they were obviously not the only ones.

In cattle, we set up a mixed-leukocyte-reaction (MLR) by mixing PBMCs from a responder animal with mitomycin-C

treated PBMCs from a genetically unmatched animal as stimulating cells (ratio 1:1) for a total of 9 days. After initial decline of total DNA content during 6 days due to cells dying in the wells, proliferation of T-cells from the responder animal was measured by an increase of the DNA content at day 9 in the control wells, correlating with polyclonal activation of T-cells (Supplementary Figure S4). Level of proliferation obtained under these control conditions was set as 100% (Figure 6D). Addition of classical MHC-II<sup>neg</sup> bovine neutrophils to the PBMCs (ratio 1:1) from the responder animal did not change the proliferative capacity. By contrast, addition of MHC-II<sup>pos</sup> neutrophils from the responder animal to PBMCs (ratio 1:1) strongly suppressed T-cells as the proliferation was completely inhibited for 3 animals, and only 16% of proliferation remained for 1 animal, as compared to control wells (Figure 6D). Decreasing the MHC-II<sup>pos</sup> neutrophils concentration, alleviated the suppressive effect (Supplementary Figure S5) showing the





**FIGURE 6 |** MHC-II<sup>pos</sup> but not MHC-II<sup>neg</sup> neutrophils exert suppression of T cells at steady state. **(A)** In the mouse an antigen-specific proliferation assay was set up with OT-II transgenic mice that carry the monoclonal population of T-cells specific for the Ova 323-329 peptide. Proliferation of splenocytes from OT-II mice activated with the Ova 323-329 peptide was set up as the maximum and compared to conditions where MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils purified from syngenic C57BL/6 mice after cell sorting (99% purity) were added with a ratio of 1:10. **(B)** The percentage of T cell proliferation after addition of MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils was calculated based on OT-II splenocytes proliferation with ova peptide only. Neutrophils were prepared from the BM of syngenic mice except for the “blood” sample as indicated on the graph. Different conditions for the assay are also indicated, with cells separated by a transwell device, or neutrophils incubated with anti-MHC-II, anti-CD11b, or isotype controls before addition to the proliferating splenocytes. **(C)** For the bovine, a mixed leucocyte reaction (MLR) was set up to assess polyclonal T cell proliferation. PBMCs from the responder animal were isolated and left untreated, while PBMCs from the stimulating animal were incubated with mitomycin C. PBMCs from the two cows were incubated at ratio of 1:1. To assess the impact of neutrophils on cattle T cell proliferation, 10<sup>5</sup> of sorted MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils from the responder animal were added to the reaction at day 4. DNA was quantified at day 6 and 9. **(D)** The proliferation was calculated by subtracting DNA values at day 9 from day 6 values. Proliferation measured in the MLR without neutrophils was defined as the reference 100% proliferation. The proliferation observed in the presence of sorted MHC-II<sup>pos</sup> or MHC-II<sup>neg</sup> neutrophils was calculated according to the DNA content (day 9–day 6) and expressed as percentage of the reference value. In one experiment, MHC-II<sup>pos</sup> neutrophils were separated from proliferating cells by a Transwell device. **(B, D)** Individual data and the median in each group are represented (3 independent experiments, n=3 pool of 10 mice, n=1 cow for transwell experiment; each represented value is the mean of technical triplicates). \*P<0.05; \*\*P<0.01; \*\*\*\*P<0.0001 (Mann Whitney non-parametric test).

dose-response effect of MHC-II<sup>Pos</sup> neutrophils on T-cells. In one experiment we could separate MHC-II<sup>Pos</sup> neutrophils from the proliferating PBMCs in a transwell device and observed that suppression was abolished, indicating that the suppressive activity of MHC-II<sup>Pos</sup> neutrophils was contact-dependent as in the mouse (Figure 6D). Therefore, both in the mouse and the bovine, a subset of MHC-II<sup>Pos</sup> neutrophils can be distinguished from classical neutrophils as displaying suppressive activity on T-cells at steady state.

## DISCUSSION

We reported here that a population of MHC-II<sup>Pos</sup> neutrophils was present in the BM reservoir, circulated in blood and was enriched in lymphoid organs, in the apparently healthy bovine and mouse. Both MHC-II<sup>Pos</sup> and MHC-II<sup>Neg</sup> neutrophils displayed the polylobed nucleus, and were undistinguishable by this gold standard of neutrophils characterization (14). Similar to classical MHC-II<sup>Neg</sup> neutrophils, the MHC-II<sup>Pos</sup> subset displayed important functions of neutrophils such as ROS production, phagocytosis, and bacterial killing. However, unlike classical MHC-II<sup>Neg</sup> neutrophils, the MHC-II<sup>Pos</sup> neutrophils were able to suppress T-cells, a function that is reported here for the first time for bovine neutrophils. Heterogeneity or plasticity of neutrophils has largely emerged in the literature in humans or mice and new models of neutrophil differential development are proposed (15). Here, mouse and bovine MHC-II<sup>Pos</sup> suppressive neutrophils were detected in the BM reservoir. They were the only ones to exert suppressive activity on T cells. They displayed a clearly distinct transcriptomic profile as compared to MHC-II<sup>Neg</sup> neutrophils. They were also highly enriched in lymphoid organs. Thus, they may represent a distinct subset, produced in the BM for rapid mobilization and regulation of T cells. On the other hand, CD11b upregulation that was observed on these neutrophils could also sign an activated state (29). Recently, CD11b<sup>hi</sup> primed neutrophils were also reported to circulate in blood in healthy mice and humans to quickly respond to danger (30). Neutrophils from healthy humans could be induced *in vitro* to exert ROS, CD11b and contact-dependent suppressive activity on T cells upon activation with specific stimuli (31). We detected MHC-II<sup>Pos</sup> neutrophils in the BM and, at least in the mouse, they were able to suppress T-cell proliferation, suggesting that they were already present as regulatory cells in the reservoir. Moreover, the regulatory neutrophils from the two species did not undergo higher apoptosis or death as compared to their classical neutrophils counterparts upon short-term *ex-vivo* stimulation with LPS, indicating they were not “older” or hyperactivated (32). In addition, MHC-II expression by MHC-II<sup>Neg</sup> neutrophils could not be induced *in vitro* by incubation with LPS (data not shown). Whether heterogenous neutrophils are released from the bone-marrow as distinct subsets under steady state conditions or correspond to activation or polarization states *in situ* remains an open debate (33) but what we observed here is that MHC-II<sup>Pos</sup> neutrophils able to regulate T cells display a specific neutrophil “phenotype” (34).

Suppressive neutrophils that may be included within a broader category of cells termed “myeloid-derived suppressor cells (MDSCs)” (35) accumulate mostly under pathological conditions. Two main branches of MDSCs have been described: the monocytic MDSCs and the granulocytic MDSCs (G-MDSCs) (35). In cancer, G-MDSCs are associated with tumor progression and escape to the immune surveillance (36). In chronic infections such as tuberculosis (37, 38) or AIDS (39), or acute inflammatory syndrome such as sepsis (40) MDSCs are targeted for new host-directed therapies. However, MDSCs, including regulatory neutrophils, are also beneficial to the host under some circumstances. Because they sustain the generation of regulatory T-cells (41) they may help avoiding graft rejection (42). They can positively regulate autoimmune disorders (43) or protect the lung from destructive inflammation during *Pseudomonas aeruginosa* or *Klebsiella pneumoniae* infections (44). They accumulate during pregnancy where they are important regulators of fetal-maternal tolerance (45) and in neonates where they could prevent overwhelming inflammation following microbial colonization (46). Recently, Aarts and colleagues demonstrated that neutrophils from healthy humans could become T-cell suppressive under specific stimulation (31). Along the same line, Fine et al. recently described that neutrophils circulate in healthy donors under several states included as “primed” cells that are first recruited to the injury site to combat infection (30). In both these studies, high levels of CD11b signed the phenotype of these neutrophils, alike the MHC-II<sup>Pos</sup> regulatory neutrophil subset that we describe here. In cattle, there is no description to date of suppressive neutrophils or G-MDSCs. In a recent report, Li and colleagues (47) identified a subset of MHC-II<sup>Pos</sup> neutrophils that accumulated in spleen during *Ostertagia ostertagi* parasitic infection in cattle and produced the suppressive cytokine IL-10. This subset was the master regulator of immune suppression in parasitized animals. We did not observe *il10* transcription in any neutrophil subset nor IL-10 production in the supernatant of neutrophil-T-cells cocultures in our study (data not shown). Whether MHC-II<sup>Pos</sup> regulatory neutrophils that we identified are related to the subset identified by Li et al. (47) remains an open question.

Regulatory neutrophils, or G-MDSCs, block T-cell proliferation through different mechanisms (14) including ROS production (48). We observed in both species that direct contact between MHC-II<sup>Pos</sup> neutrophils and T-cells was necessary to induce suppression, as was also reported for G-MDSCs. Formation of a synapse is believed to greatly enhance suppression especially when the delivery of short-lived molecules such as H<sub>2</sub>O<sub>2</sub> is instrumental (35). Interestingly, we observed here a significantly higher production of total ROS upon chemical stimulation by MHC-II<sup>Pos</sup> neutrophils as compared to their MHC-II<sup>Neg</sup> counterparts which did not translate into higher killing of *E. coli*. Pliyev and colleagues demonstrated that NADPH dependent ROS were required for the expression of MHC-II in human neutrophils activated by GM-CSF and IFN $\gamma$  (49). Whether the higher ROS production recorded in our study is linked to the suppression mechanism remains to be investigated. In both the mouse and the bovine (data not shown), MHC-II was also involved in the mechanism, as blocking this molecule partially relieved the suppression of T-cell proliferation, emphasizing the need for



synapse formation between the T-cell and the regulatory neutrophil. We established in the past the important role of CD11b expressed by neutrophils in phagocytic synapse and crosstalk with other cells, as well as specific signaling pathways (50, 51). In the present study, we observed that CD11b was also involved in the suppression mechanism in mice, as reported for human neutrophils acquiring suppressive activity upon *in vitro* stimulation. Direct CD11b-dependent interactions between neutrophils and T cells was required for suppression but additional molecules such as ICAM-3 on T-cells may also function as ligands for CD11b (31). Similarly, our results in the mouse model suggested that a synapse with T-cells, involving CD11b and MHC-II on the surface of neutrophils, was necessary to exert suppression. However, suppression by MHC-II<sup>Pos</sup> neutrophils clearly involved other mechanisms that we will investigate in the near future. In that respect, surface expression and up-regulated gene transcription of *PD-L1* by mouse regulatory neutrophils is an interesting avenue. This molecule is an important immune check-point and a favorite target for cancer treatment. MDSCs express high levels of CD274 (52) which is involved in T-cell suppression. Of note, CD14<sup>Pos</sup> monocytes expressing CD274 accumulate in blood of cattle infected by *Mycoplasma bovis* (53), a strongly immunosuppressive pathogen causing antibiotic resistant mastitis and other diseases. CD274 is now a target for host-directed therapies of cattle infected by this pathogen (54). We could not investigate CD274 expression by cattle MHC-II<sup>Pos</sup> regulatory neutrophils due to a lack of available Ab. We did not observe differences at the mRNA level between the two subsets (data not shown) but whether such differences exist at the protein level remains to be investigated.

To conclude, comparative analysis of mouse and bovine species allowed us to characterize a new subset of regulatory neutrophils that are able to suppress T cells. In the near future, we will investigate how these cells behave during clinical conditions in cattle such as mastitis, which remains one of the most important issue in dairy farming. We believe that such studies are of utmost importance to better understand the physiopathology of this disease, especially during chronic infections that remain difficult to treat. Our findings could lead to the discovery of new biomarkers and development of innovative host-directed therapies (54) targeting regulatory neutrophils for more effective clearance of pathogens and better control of mammary gland inflammation and damage.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

## ETHICS STATEMENT

The animal study was reviewed and approved by Val de Loire Ethics Committee for Animal Experimentation.

## AUTHOR CONTRIBUTIONS

MR designed and did most of the experiments for cattle neutrophils and ED-D for mouse neutrophils. They analyzed data and prepared all manuscript figures. YV performed the flow cytometry analysis and sorting of mouse and cattle neutrophils. PC helped with the mixed leukocyte reaction in cattle. PG helped with transcriptomic analysis, critically analyzed the data, and revised the manuscript. PR brought valuable expertise on cattle neutrophil purification methods, critically analyzed the data, and revised the manuscript. NW and AR obtained grants, supervised all aspects of the work, critically analyzed the data, and wrote the manuscript. AR also contributed to the important experiments in cattle. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.625244/full#supplementary-material>

**Supplementary Table 1** | Antibodies used in the study. The specificity, origin, clone number and commercial provenance of all antibodies used in the study are listed.

**Supplementary Table 2** | Sequences of primers used in this study. Primers were designed using Geneious software, in intron-spanning regions when possible. The



annealing temperature was set at 60 and 62°C for bovine and mouse samples respectively. Housekeeping genes used as the reference to calculate  $\Delta\Delta CT$  for each species are indicated in the yellow boxes and weakly expressed genes that were removed from the Principal Component Analysis presented in **Figure 4** are indicated in the grey boxes.

**Supplementary Figure 1** | Isotype controls for neutrophil diversity analysis in mouse bone marrow and cattle blood. Mouse and bovine neutrophils were labeled as described in **Figure 2**. Appropriate isotype controls for all antibodies in each experiment were used to correctly set the analysis and sorting gates. Dot plots from one representative animal are depicted (3 independent experiments, n=4 mice, n=6 for bovine).

**Supplementary Figure 2** | Gating strategy for neutrophil diversity analysis in all organs. Mouse and bovine neutrophils were labeled as described in **Figure 2**. Similar procedures were set up with isotype controls for all antibodies in each experiment to correctly set the analysis gates. Dot plots from one representative animal are depicted (3 independent experiments, n=4 mice, n=6 for bovine).

**Supplementary Figure 3** | Gating strategy for analysis of phagocytosis and ROS production by neutrophils. Neutrophils were labeled and sorted as described in **Figures 2** and **S1**. **(A)** After purification by cell sorting from the BM (mouse) or blood (bovine) phagocytosis by MHC-IFN $\gamma$  or MHC-IFN $\alpha$  neutrophils was assessed using pHrodo E.coli bioparticles with or without previous treatment with cytochalasin D. Dot plots from one representative animal are depicted (3 independent experiments, n=3 pool of 10 mice, n=3 for bovine). **(B)** Oxidative stress was measured in MHC-IFN $\alpha$  and MHC-IFN $\gamma$  sorted neutrophils using the CellROX Orange probe that reacts with all ROS species. Cells were activated with TBHP or incubated with medium

alone and levels of ROS were measured by flow cytometry among the live cells (unstained with eFluor780 viability dye). Dot plots from one representative animal are depicted (3 and 4 independent experiments for mice and cattle respectively, n=3 pool of 10 mice, n=5 for bovine).

**Supplementary Figure 4** | Kinetics of bovine Mixed Leukocyte Reaction and analysis of neutrophil suppressive activity. **(A)** PBMCs from the responder animal were isolated and left untreated, while PBMCs from the stimulating animal were incubated with mitomycin C to block their proliferation. PBMCs from the two cows were incubated at ratio of 1:1. Sorted MHC-IFN $\alpha$  or MHC-IFN $\gamma$  neutrophils from the responder animal were added to the reaction at day 4. **(B)** DNA was quantified at different time points with CyQUANT Cell Proliferation Assay tests according to manufacturer's instruction and fluorescence was read at 530nm. DNA extracted from PBMCs cultivated separately decreased along the assay indicated the absence of proliferation (dotted lines). In the MLR reaction, while DNA content declined between day 1 and 6, PBMCs proliferation could be measured between day 6 and 9 (black). The effect of adding sorted MHC-IFN $\alpha$  neutrophils (blue) or MHC-IFN $\gamma$  neutrophils (orange) to the proliferating cells could then be measured. One representative experiment is shown and data represent the mean  $\pm$  SEM of technical triplicates. Four independent experiments were conducted with different pairs of cows.

**Supplementary Figure 5** | Bovine MHC-IFN $\alpha$  neutrophil suppressive activity is dose-dependent. The suppressive assay was performed as described in **Figures 6** and **S4**. At day 4,  $1 \times 10^5$ ,  $5 \times 10^4$  or  $1 \times 10^4$  purified MHC-IFN $\alpha$  neutrophils from the responder animal were added to the MLR reaction. One experiment was performed (technical duplicates are depicted).

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Mycobacterial Infection of Precision-Cut Lung Slices Reveals Type 1 Interferon Pathway Is Locally Induced by *Mycobacterium bovis* but Not *M. tuberculosis* in a Cattle Breed

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Tuberculosis exacts a terrible toll on human and animal health. While *Mycobacterium tuberculosis* (Mtb) is restricted to humans, *Mycobacterium bovis* (Mb) is present in a large range of mammalian hosts. In cattle, bovine TB (bTB) is a noticeable disease responsible for important economic losses in developed countries and underestimated zoonosis in the developing world. Early interactions that take place between mycobacteria and the lung tissue early after aerosol infection govern the outcome of the disease. In cattle, these early steps remain poorly characterized. The precision-cut lung slice (PCLS) model preserves the structure and cell diversity of the lung. We developed this model in cattle in order to study the early lung response to mycobacterial infection. *In situ* imaging of PCLS infected with fluorescent Mb revealed bacilli in the alveolar compartment, in adjacent or inside alveolar macrophages, and in close contact with pneumocytes. We analyzed the global transcriptional lung inflammation signature following infection of PCLS with Mb and Mtb in two French beef breeds: Blonde d'Aquitaine and Charolaise. Whereas, lungs from the Blonde d'Aquitaine produced high levels of mediators of neutrophil and monocyte recruitment in response to infection, such signatures were not observed in the Charolaise in our study. In the Blonde d'Aquitaine lung, whereas the inflammatory response was highly induced by two Mb strains, AF2122 isolated from cattle in the UK and Mb3601 circulating in France, the response against two Mtb strains, H37Rv, the reference laboratory strain, and BTB1558, isolated from zebu in Ethiopia, was very low. Strikingly, the type I interferon pathway was only induced by Mb but not Mtb strains, indicating that this pathway may be involved in mycobacterial virulence and host tropism. Hence, the PCLS model in cattle is a valuable tool to deepen our understanding of early interactions between lung host cells and mycobacteria. It revealed striking differences



between cattle breeds and mycobacterial strains. This model could help in deciphering biomarkers of resistance vs. susceptibility to bTB in cattle as such information is still critically needed for bovine genetic selection programs and would greatly help the global effort to eradicate bTB.

**Keywords:** cattle, *Mycobacterium bovis*, ex vivo, precision cut lung slices, alveolar macrophages, type I interferon

## INTRODUCTION

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (Mb) remains one of the most challenging infections to control in cattle. Because of its zoonotic nature, this pathogen and its associated noticeable disease in cattle are under strict surveillance and regulation in the European Union. When bTB cases are detected through surveillance, culling of these reactor cattle is mandatory. In spite of intensive eradication campaigns, bTB is still prevalent in European cattle (1, 2) and has significant economic, social, and environmental implications. Since 2001, France is an officially bTB-free country, a status that was achieved through costly surveillance programs. However, each year, around 100 Mb foci of infection are identified (3), with certain geographical areas showing a constant rise in disease prevalence since 2004.

bTB eradication is an unmet priority that faces two major difficulties: the persistence of undetected infected animals in herds because of the lack of diagnostic sensitivity and the risk of transmission from infected sources (4). Moreover, the poor understanding of bTB pathophysiology in cattle and the lack of correlates of protection are substantial knowledge gaps that must be resolved so as to better tackle the disease (DISCONTTOOLS, <https://www.discontools.eu/>).

Both Mb and *Mycobacterium tuberculosis* (Mtb) belong to the same genetic complex. Mtb is responsible for tuberculosis (TB) in humans, which displays similar features with bTB. It is estimated that one-third of the global human population are latently infected with Mtb, which kills 1.4 million people each year (5). Despite the high degree of identity that Mtb and Mb share both at the genetic level as well as during the infection process, the two pathogens display distinct tropism and virulence depending on the host. While Mb is highly virulent and pathogenic for cattle and a range of other mammals, Mtb is restricted to sustain in humans. An experimental infection of cattle with the widely used Mtb laboratory strain H37Rv, which was genome-sequenced in 1998 (6), shows a strong attenuation compared to Mb (7, 8). However, the natural infection of cattle with Mtb has been reported, and the strain Mtb BTB1558 was once such a case, isolated from a zebu bull in Ethiopia (9, 10). In comparison to the original UK Mb strain AF2122/97, the first genome-sequenced Mb isolate (11, 12), the Mtb strain BTB1558 displayed a much lower virulence in European cattle (13).

The Mb strains that circulate in France today are phylogenetically distant from the UK Mb reference strain. While AF2122 belongs to the European 1 clonal complex (14), the European 3 clonal complex is widespread in France, (15). The Eu3 genetic cluster is composed of field strains that

share the SB0120 spoligotype with the attenuated Bacillus-Calmette-Guerin (BCG) vaccine strain (16, 17). In our study, we used Mb3601 as the representative strain of this widespread French cluster. Originally, Mb3601 was isolated from the tracheobronchial lymph node of an infected bovine in a bTB highly enzootic area in France (16). However, despite the widespread circulation in its original area, nothing is known today of the pathophysiology of Mb3601 infection.

Indeed greater knowledge is available on Mtb infection process and disease development both in humans and mouse models compared to Mb infection in cattle. With both mycobacteria, the alveolar macrophage (AMP) is the frontline cell that first presents the first niche for mycobacteria entering the lung, and the role of the AMP in early-stage infection is well established (8). Both Mtb and Mb have established their lifestyle in AMPs: they can escape its bactericidal mechanisms and multiply within this niche. During the infection process, bacilli disseminate to different anatomical sites and establish new infection foci both in the lungs and secondary lymphoid organs (18, 19). During Mtb infection, lung epithelial cells also play key roles in host defense [reviewed in (20–22)]. Type II pneumocytes are infected by Mtb (23) and produce pro-inflammatory cytokines which augment the AMP innate resistance mechanisms (24). The role of type II pneumocytes during Mb infection in cattle is not well known. Most of the available knowledge on the role of bovine macrophages (MPs) during Mb infection also comes from studies conducted with monocytes sampled from blood and derived as MPs during *in vitro* culture (25, 26).

In our study, we wanted to investigate the bovine innate response following Mb or Mtb infection in a preserved lung environment to allow the resident lung cells to interact with bacilli and crosstalk. Precision cut lung slices (PCLS) are an experimental model in which resident lung cell types are preserved and remain alive for at least 1 week (27). The tissue architecture and the interactions between the different cells are maintained. PCLS have already been validated for the study of various respiratory pathogens (27–29). In chicken PCLS, mononuclear cells are highly motile and actively phagocytic (30). This model is well designed to study complex interactions taking place early after the host–pathogen encounter. During Mb infection in cattle, important differences in the production of key proinflammatory cytokines such as IFN $\gamma$  or TNF $\alpha$  by peripheral blood mononuclear cells are observed, depending on the clinical status of the animal. Interestingly, such differences are observed at early time points (31), indicating that the innate phase of the host response is key to the establishment of the pathological outcome of the infection.



Therefore, the PCLS model is ideally suited to investigate early host–pathogen interactions in the bovine lung during Mb infection and may help to find clues to the impact of the innate response on the outcome of infection. This model, which fully mimics the early environment of the bacillus entering the lung (compared to monocyte-derived MPs), may also aid in understanding the molecular basis of mycobacterial host preference (32). To this end, we decided to compare four mycobacterial strains: two Mtb species—namely, the Mtb H37Rv reference strain for human TB and the cattle derived Mtb BTB1558—and two Mb species—namely, Mb AF2122 as representative of the EU1 clonal complex and Mb3601 as the hallmark EU3 strain. Since the host genetic background also has a profound impact on the outcome of bTB disease (33), we decided to compare PCLS from two prevalent beef breeds in France—Charolaise and Blonde d'Aquitaine—and conducted a thorough characterization of the lung responses to Mb and Mtb during *ex vivo* infection. The PCLS allowed us to decipher important differences in the transcriptomic and cytokine profile during the innate response to infection, depending both on the breed, i.e., between Blonde d'Aquitaine and Charolaise cows, and on the mycobacterial species, i.e., between Mtb and Mb.

## MATERIALS AND METHODS

### Animal Tissue Sampling

Lungs from 15 Blonde d'Aquitaine and nine Charolaise cows were collected post-mortem at a commercial abattoir. The animals were between 3 and 11 years old and originated from eight different French departments where no recent bTB outbreak had been noticed (**Supplementary Figure 1**). No ethical committee approval was necessary as no animal underwent any experimental procedure. After slaughter by professionals following the regulatory guidelines from the abattoir, the lungs from each cow were systematically inspected by veterinary services at the abattoir. The origin of each animal was controlled, and its sanitary status was recorded on its individual passport: the animals were certified to be free of bTB, leucosis, brucellosis, and infectious bovine rhinotracheitis.

### Bacterial Strains and Growth Conditions

Strains Mb AF2122/97 and Mb MB3601 had previously been isolated from infected cows in Great Britain and France, respectively (12, 15). The Mb3601-EGFP fluorescent strain was derived by electroporation with an integrative plasmid expressing EGFP and selected with Hygromycin B (50 µg/ml) (Sigma, USA) as described previously (34). Mtb BTB1558 had been previously isolated from a zebu bull in Ethiopia (13). Bacteria were grown in Middlebrook 7H9 broth (Difco, UK) supplemented with 10% BBLTM Middlebrook albumin–dextrose–catalase (BD, USA) and 0.05% Tween 80 (Sigma-Aldrich, St Louis, USA). At mid-log phase, the bacteria were harvested, aliquoted, and stored at –80°C. Batch titers were determined by plating serial dilutions on Middlebrook 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase (BD, USA), with 0.5% glycerol or 4.16 g/L sodium pyruvate (Sigma, USA) added for Mtb or Mb strains, respectively. The plates were incubated at 37°C for 3–4 weeks

(H37Rv, BTB558, and AF2122) and up to 6 weeks for Mb3601 before colony-forming unit (CFU) enumeration. The inocula were prepared from one frozen aliquot (titer determined by CFU enumeration) that was thawed in 7H9 medium without glycerol and incubated overnight at 37°C. After centrifugation for 10 min at 3,000 × g, the concentration was adjusted to 10<sup>6</sup> CFU/ml in RPMI medium.

### Obtention and Infection of Precision-Cut Lung Slices

PCLS were obtained from fresh lungs using a tissue slicer, MD 6000 (Alabama Research and Development). For each animal, the right accessory lobe was filled *via* the bronchus with RPMI containing 1.5% low-melting-point (LMP) agarose (Invitrogen) warmed at 39°C. After 20 min at 4°C, the solidified lung tissue was cut in 1.5-cm slices with a scalpel. A 0.8-mm diameter-punch was used to obtain biopsies that were placed in the microtome device of the Krumdieck apparatus, filled with cold phosphate-buffered saline (PBS), and 100-µm-thick PCLS were cut. One PCLS was introduced in each well of a 24-well plate (Nunc); 1 ml of RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco), 2 mM L-glutamine (Gibco), and PANTA™ antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton Dickinson) was added to the well, and the plate was incubated at 37°C with 5% CO<sub>2</sub>. The medium was changed every 30 min during the first 2 h to remove all traces of LMP agarose. At 24 h later, after the last medium change, ciliary activity was observed under a microscope to ensure tissue viability.

The PCLS were infected for 2 days with 10<sup>5</sup> CFU of Mb or Mtb strains. As indicated, the PCLS were either fixed in formalin for imaging or lysed with a Precellys in lysing matrix D tubes in 800 µl Tri-reagent for RNA extraction. The bacillary load of each strain present in the PCLS was compared after the transfer of the PCLS to a new plate at 1 day after infection (dpi), two washes in 1 ml of PBS, and homogenization in 1 ml of PBS in lysing matrix D tubes (MP Biomedicals) with a Precellys (Ozyme). To determine CFUs, serial dilutions were plated as described above.

### Alveolar Macrophages

To harvest alveolar macrophages (AMPs) from Blonde d'Aquitaine cows, broncho-alveolar lavages (BAL) were performed on the left basilar lobe of the lung at a local abattoir after culling the animal. The lobe was filled with 2 × 500 ml of cold PBS containing 2 mM EDTA (Sigma-Aldrich). After the massage, the BAL was collected and transported at 4°C to the laboratory. BAL was filtered with a 100-µm cell strainer (Falcon) and centrifuged for 10 min at 300 × g. The cells were washed in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM L-glutamine (Gibco), and PANTA™ Antibiotic Mixture. Then, 10<sup>7</sup> BAL cells per milliliter were suspended in 90% FCS and 10% dimethyl sulfoxide (Sigma-Aldrich) and cryopreserved in liquid nitrogen. At 1 day before infection, the BAL cells were thawed at 37°C, washed in complete RPMI medium, and transferred to a 75-cm<sup>2</sup> culture flask with a ventilated cap. After 2 h at 37°C and 5% CO<sub>2</sub>, non-adherent cells were removed, and adherent AMPs were incubated 2 × 10 min



at 4°C with 10 ml of cold PBS to detach and enumerate them in a Malassez chamber. Then,  $5 \times 10^5$  AMPs/well were distributed in a 24-well plate and incubated overnight at 37°C and 5% CO<sub>2</sub>. The medium was changed once, and AMPs were infected with Mb3601 or Mtb H37Rv at a multiplicity of infection (MOI) of 1. At 6 and 24 h post-infection, the supernatants were filtered through a 0.2- $\mu$ m filter, and the cells were lysed in 800  $\mu$ l of Tri-reagent for RNA extraction. The MOI was checked by CFU determination at 24 h after infection.

## Cell Supernatant Collection and Lactate Dehydrogenase Assay

In order to evaluate cytotoxicity, supernatants from infected PCLS or AMPs were passed through a 0.2- $\mu$ m filter at indicated time points, and cells were lysed in 1 ml of lysis buffer (5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, Triton 1%, pH 7.4), containing anti-proteases (Roche), in a lysing matrix D tube, with a Precellys apparatus. The homogenates were clarified by centrifugation for 10 min at  $10,000 \times g$ , filtered through 0.2  $\mu$ m, and collected on microplates. The cytotoxicity of infection in PCLS was assessed using the Non-radioactive Cytotoxicity Assay kit (Promega) according to the manufacturer's instructions. The cytotoxicity was calculated as cytotoxicity (%) =  $[\text{OD}_{490} \text{ of lactate dehydrogenase (LDH) in the supernatant}] / (\text{OD}_{490} \text{ of LDH in the supernatant} + \text{OD}_{490} \text{ of LDH in the PCLS homogenates}) \times 100$ .

## Immunohistochemistry on PCLS

The infected PCLS were fixed 24 h at 4°C with 4% formalin and then transferred to a 48-well culture plate in PBS. All steps that will be described below were done under gentle agitation at room temperature (RT). The PCLS were incubated for 2 h with 100  $\mu$ l of PBS, 0.25% Triton X-100, and 10% horse serum for permeabilization and saturation (saturation buffer). They were incubated overnight at 4°C with primary Ab (anti-bovine MHCII clone MCA5655 from BioRad and anti-bovine pancytokeratine clone BM4068 from Acris) diluted in saturation buffer. The PCLS were washed four times with 300  $\mu$ l of PBS (two times for 5 min and then two times for 10 min) and then incubated for 3 h with fluorescent-conjugated secondary antibodies diluted in saturation buffer (goat anti-mouse IgG1-APC and goat anti-mouse IgG2a A555 from Invitrogen). The PCLS were washed four times with 300  $\mu$ l of PBS (two times for 5 min and then two times for 10 min), transferred on cover slides which were mounted with Fluoromount-G<sup>TM</sup> mounting medium containing DAPI (Invitrogen), and sealed with a transparent nail polish. Z-stack imaging was performed at  $\times 63$  enlargement with a confocal microscope (LEICA) and analyzed with LAS software. The presence/absence of Mb and number of macrophages per alveoli were numerated by eye at the confocal microscope, with one person counting and the other confirming and reporting the data.

## Quantification of Cytokines and Chemokines Released by PCLS and AMPs

The cytokine and chemokine levels produced by PCLS after 2 dpi were assessed in a Multiplex assay in supernatants (dilution 1:2) with MILLIPLEX<sup>®</sup> Bovine cytokine/chemokine panel 1 (BCYT1-33K-PX15, Merck) according to the manufacturer's

instructions. IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-17A, IL-36RA (IL-1F5), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), TNF $\alpha$ , and VEGF-A were measured. Data were acquired using a MagPix instrument (Luminex) and analyzed with Bio-Plex Manager software (Bio-Rad). IL-8 was out of range in the Multiplex, so we performed a sandwich ELISA with the following references: goat anti-bovine interleukin-8 Ab AHP2817, recombinant bovine interleukin-8 BPB039, and goat anti-bovine interleukin-8 Ab conjugated to biotin AHP2817B (all from Bio-Rad), following the protocol according to the manufacturer's instructions.

## RNA Extraction and Gene Expression Analysis

The total RNA from two pooled PCLS was extracted using a MagMAX<sup>TM</sup>-96 Total RNA isolation kit (ThermoFisher). For AMPs, we used the Nucleospin RNA isolation kit (Macherey Nagel). After DNase treatment (ThermoFisher or Macherey Nagel), the mRNAs were reverse-transcribed with iScript<sup>TM</sup> Reverse Transcriptase mix (Biorad) according to the manufacturer's instructions. The primers (Eurogentec; **Supplementary Table 1**) were validated, using a serially diluted pool of cDNA mix obtained from bovine lung, lymph nodes, blood, and bone marrow, with a LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche). Gene expression was then assessed with the BioMark HD (Fluidigm) in  $96 \times 96$ -well integrated fluidic circuit plate according to the manufacturer's instructions. The annealing temperature was 60°C. The data were analyzed with Fluidigm RealTime PCR software to determine the cycle threshold (Ct) values. The messenger RNA (mRNA) expression was normalized to the mean expression of three housekeeping genes (*PPIA*, *GAPDH*, and *ACTB*) to obtain the  $\Delta$ Ct value. For each animal, values from infected PCLS were normalized to the uninfected PCLS gene expression ( $\Delta\Delta$ Ct value and relative quantity =  $2^{-\Delta\Delta\text{Ct}}$ ). Principal component analysis (PCA) was performed using  $\Delta\Delta$ Ct values in R studio (version 1.1.456, ©2009–2018 RStudio, PBC) using the FactoMineR packages (version R 3.5.3).

## Statistical Analysis

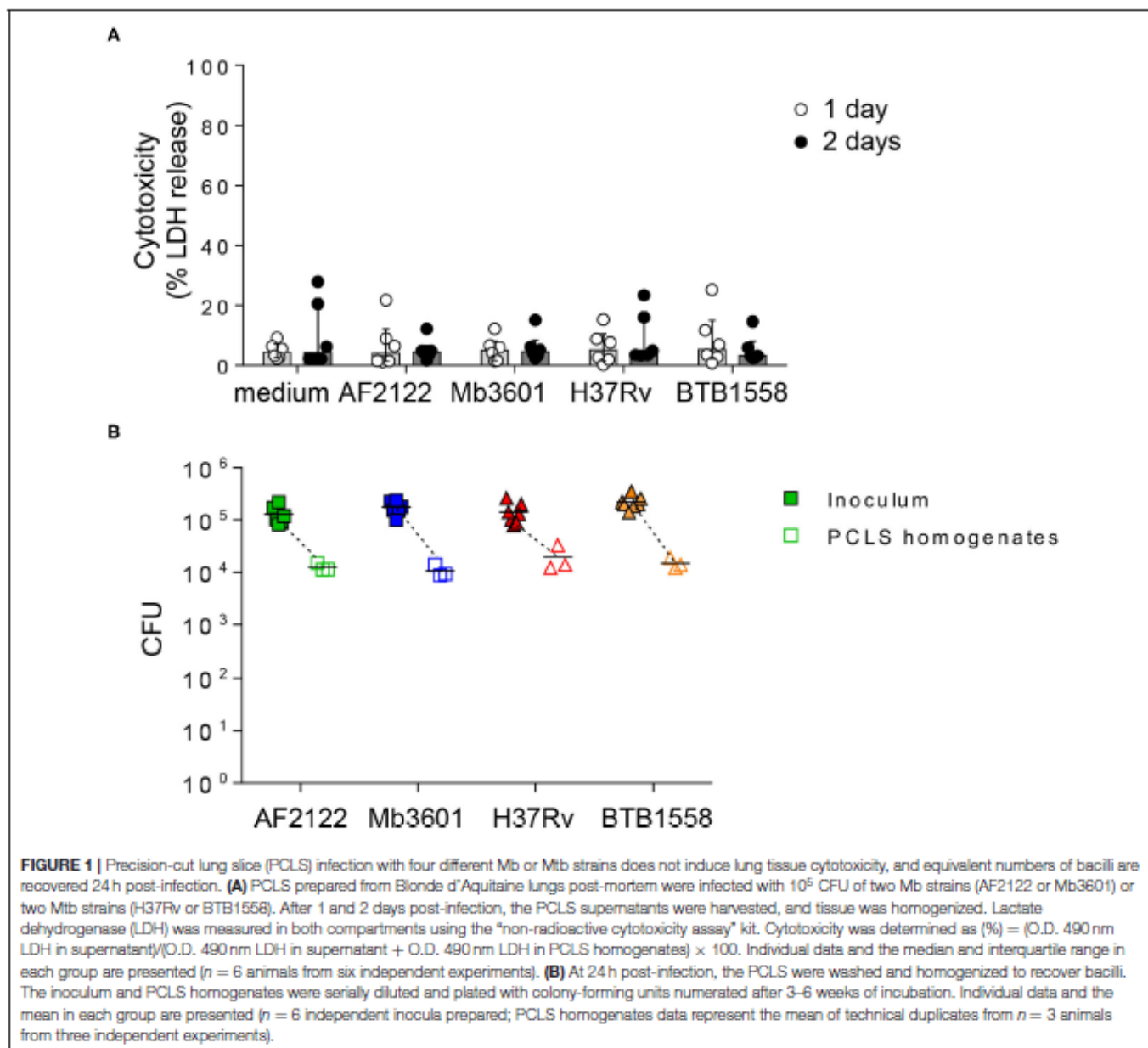
The individual data and the median and interquartile range are presented in the figures, except for **Figure 2** where the mean and standard error of the mean (SEM) are presented. Statistical analyses were performed with Prism 6.0 software (GraphPad). Analyses were performed on data from two to six independent experiments, with two-way ANOVA or Wilcoxon non-parametric tests for paired samples used. The represented *p*-values were \**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001.

## RESULTS

### *Ex vivo* Infection With Mycobacteria of Live Bovine Lung Tissue in PCLS Allows Bacilli Uptake by AMPs and Their Recruitment to the Alveoli

The early events of bTB pathophysiology in the bovine lung remain poorly defined due to the complexity of biocontained experimental infection in large animals. Since PCLS have been



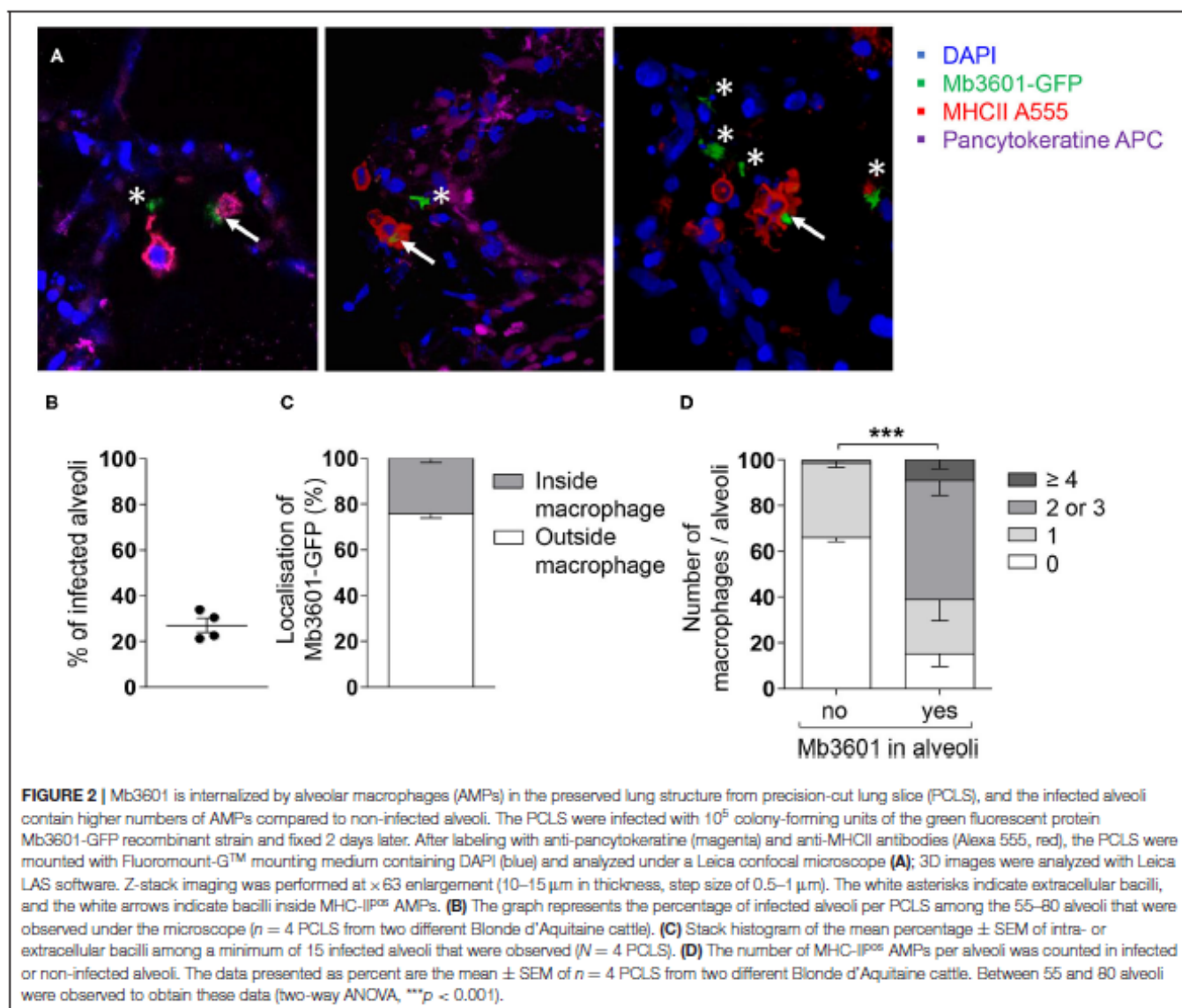


used to study viral respiratory infections in the bovine (27), we decided to use this model to assess early events taking place following entry of Mb into the lung. We infected bovine PCLS obtained *ex vivo* with the four mycobacterial strains: Mb AF2122, Mb3601, Mtb H37Rv, or BTB1558.

We first monitored tissue cytotoxicity at 1 and 2 dpi using a LDH release assay. The mean percentage of cytotoxicity remained below 10%, and no difference was observed between infected and non-infected PCLS (Figure 1A). The ciliary activity from the PCLS bronchial cells monitored every day under a light microscope remained vigorous and stable after infection (data not shown). We calibrated our model and inocula to use  $10^5$  CFUs for each of the four different strains. We analyzed CFUs still present in PCLS at 24 h later and observed an equivalent

1 log decrease for all strains (Figure 1B). This indicated an equivalent infection by all strains, allowing them to be directly compared. Therefore, with a similar bacterial load and excellent tissue viability in all experimental conditions, we validated PCLS as a model to study the early events taking place in the bovine lung after infection with mycobacteria.

In order to visualize the interactions taking place between bacilli and lung cells, we infected the PCLS with a fluorescent version of the Mb3601 strain, and at 1 and 2 dpi, we analyzed the cells by *in situ* immunohistochemistry. The lung structure was visualized by DAPI and pancytokeratine staining, and we used confocal microscopy to image 10–15- $\mu$ m sections and localize Mb3601-EGFP (Figure 2A). We observed Mb in  $27 \pm 3\%$  of PCLS alveoli (Figures 2A,B) and almost always in



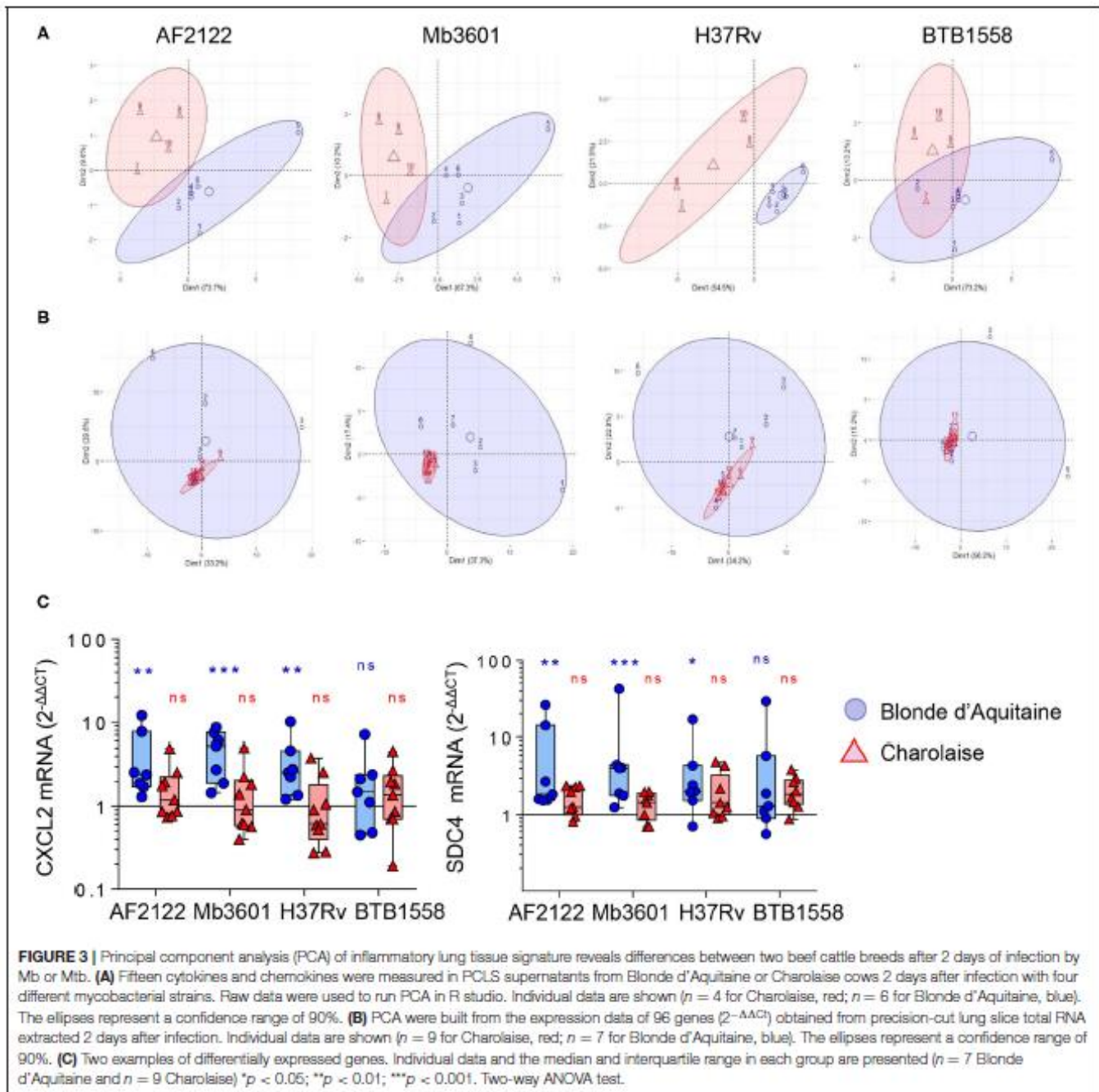
close contact with large MHC-II-positive AMPs. The bacilli were localized outside AMPs in  $76 \pm 2\%$  observations and resided intracellularly in AMPs in  $24 \pm 2\%$  (Figures 2A,C and Supplementary Video 1). Interestingly, the number of AMPs per alveoli differed upon bacilli presence or absence (Figure 2D). In uninfected PCLS, lung alveoli generally contained one AMP (data not shown). However, in Mb-infected PCLS, we either observed no AMPs in  $66 \pm 2\%$  of alveoli or one AMP in  $33 \pm 2\%$  of alveoli in the absence of any Mb. On the contrary, the number of AMPs significantly increased in alveoli where at least one Mb was observed (Figure 2D,  $p < 0.001$ ). The number of AMPs varied among infected alveoli, with  $24 \pm 9\%$  containing one AMP,  $52 \pm 6\%$  containing two or three AMPs, and  $9 \pm 4\%$  containing more than four AMPs. Such observations indicated that, during the 2 days of infection, AMPs were recruited from one alveolus to another in response to signals linked to Mb infection. In conclusion, even though Mb infection

was performed *ex vivo*, bacilli were observed in the alveoli, close or inside their target host cell, i.e., the AMP. Moreover, the PCLS model was physiological enough to allow AMPs to crawl in response to signals linked to bacilli entry.

### The Lung Response to Mycobacterial Infection Vastly Differs Between Blonde d'Aquitaine and Charolaise Cows

Two bovine beef breeds are widely used in France: Blonde d'Aquitaine and Charolaise. We decided to compare how these two breeds respond to mycobacterial infection, using our PCLS system. We measured 15 cytokines and chemokines secreted by the lung tissue at 2 dpi with the four mycobacterial strains and performed a PCA. As depicted in Figure 3A, the PCA revealed important differences in the immune response of the lung tissue between the two breeds. The group samples clearly

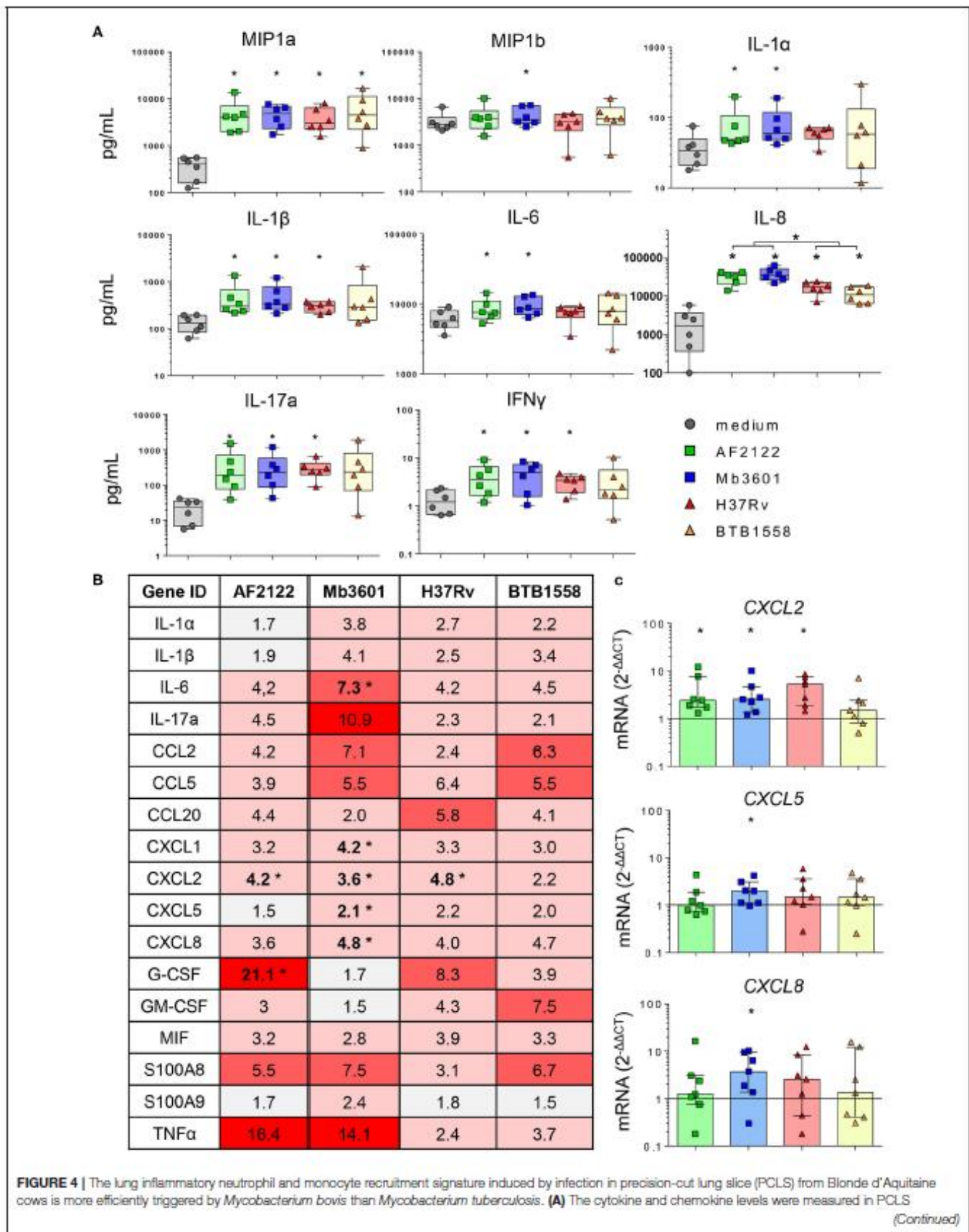




plotted apart, and their ellipses showed either a small overlay (AF2122 and Mb360A) or no overlay at all (H37Rv). The results for the BTB1558 group showed less clustering of samples due to higher individual variations. We then extracted total RNA from PCLS after 1 or 2 dpi and analyzed the expression of 96 genes related to innate immunity and inflammation (see the full list in **Supplementary Table 1**). The RT-qPCR data were normalized and expressed as fold change compared to uninfected PCLS control for each cow. Gene expression was higher at 2 days after infection compared to that at 1 dpi (data not shown). We

therefore decided to focus our analysis on this 2-dpi time point. Remarkably, the transcriptomic signature induced by infection was very low for the Charolaise breed, whichever mycobacterial strain was used, which explains the clustering of Charolaise samples (**Figure 3B**). Increasing the inoculum in the Charolaise PCLS up to  $5 \times 10^6$  CFU did not induce gene expression (**Supplementary Figure 2**). The response of the lung tissue to mycobacterial infection in Blonde d'Aquitaine was very different compared to that in Charolaise as revealed by a PCA (**Figure 3B**). Whereas, in PCLS from Charolaise the gene expression from





**FIGURE 4** | supernatant by Multiplex ELISA 2 days after infection with two Mb or two Mtb strains. Individual data and the median and interquartile range in each group are presented ( $n = 6$  cows). **(B)** Table of the mean of fold change ( $2^{-\Delta\Delta CT}$ ) for each group ( $n = 7$  cows) of 17 major genes involved in neutrophil and monocyte recruitment and inflammation. The graduated red box coloring represents levels of gene expression, and the asterisks mark significant differences compared to non-infected controls. **(C)** *CXCL2*, *CXCL5*, and *CXCL8* gene expression at 2 days post-infection. Individual data and the median and interquartile range in each group are presented ( $n = 7$  cows). **(B,C)** \* $p < 0.05$  (Wilcoxon nonparametric test).

infected and non-infected controls clustered, in PCLS from Blonde d'Aquitaine, the gene expression levels were significantly more dispersed after infection compared to those of controls (Figure 3B). We compared the individual gene expression between the two breeds for a number of genes. For instance, both the *CXCL2* chemokine and the mycobacteria receptor syndecan 4 *SDC4* were significantly upregulated after PCLS infection with AF2122, Mb3601, or H37Rv in Blonde d'Aquitaine, but not in Charolaise (Figure 3C). Our data altogether revealed important differences in the early lung response to mycobacterial infection, depending on the breed of the animals, that could be measured both at the gene expression and protein production level in the PCLS system.

### The Overall Inflammation Signature in the Lung Tissue Is Triggered More Efficiently by *M. bovis* Than *M. tuberculosis*

We then focused our analysis on Blonde d'Aquitaine to determine how the lung tissue responded to different mycobacterial strains. We analyzed 15 cytokines and chemokines produced in the PCLS supernatants 2 days following an infection. No IL-4 was detected, and the production of TNF $\alpha$ , IL-36RA, IL-10, VEGFA or MCP-1 was not different between infected PCLS and controls (Supplementary Figure 3A). We observed that *ex vivo* infection of PCLS with mycobacteria triggered an inflammatory response that contrasted between the strains (Figure 4A). At the protein level, the Mtb strain BTB1558 induced the most heterogeneous response, and due to high individual variation, differences in chemokine/cytokine production between infected PCLS and controls only reached a statistical significance for MIP-1a (CCL3) and IL-8 (Figure 4A). These two inflammatory mediators were also strongly induced by all strains. IL-17A, IL-1 $\beta$ , and IFN $\gamma$  were efficiently induced by mycobacterial infection, and no significant difference was observed between Mtb and Mb. By contrast, IL-6 and IL-1 $\alpha$  were significantly induced after Mb, but not Mtb, infection, and IL-8 production was also significantly higher after Mb than Mtb infection (Figure 4A). The only strain able to induce a significant production of MIP-1b was Mb3601. We then analyzed the inflammatory transcriptomic signature using a panel of 17 genes involved in monocyte/macrophage and neutrophil recruitment (Figure 4B). A number of these genes was significantly upregulated upon PCLS infection even though significant differences were not always reached due to inter-individual variation. Remarkably, Mb3601 induced the strongest inflammatory response, with five out of 17 genes significantly upregulated compared to non-infected controls. Focusing on chemokines involved in neutrophil recruitment, we observed that *CXCL2* expression was induced by all strains—except

BTB1558—whereas *CXCL1*, *CXCL5*, and *CXCL8* were only upregulated by Mb3601 (Figures 4B,C). *IL-6* expression was also high after Mb3601 infection. Therefore, the *ex vivo* infection of PCLS efficiently triggered signals involved in monocyte and neutrophil recruitment. Infection by Mb strains, more specifically the Mb3601 strain circulating in France, triggered inflammation in the bovine lung more efficiently than Mtb.

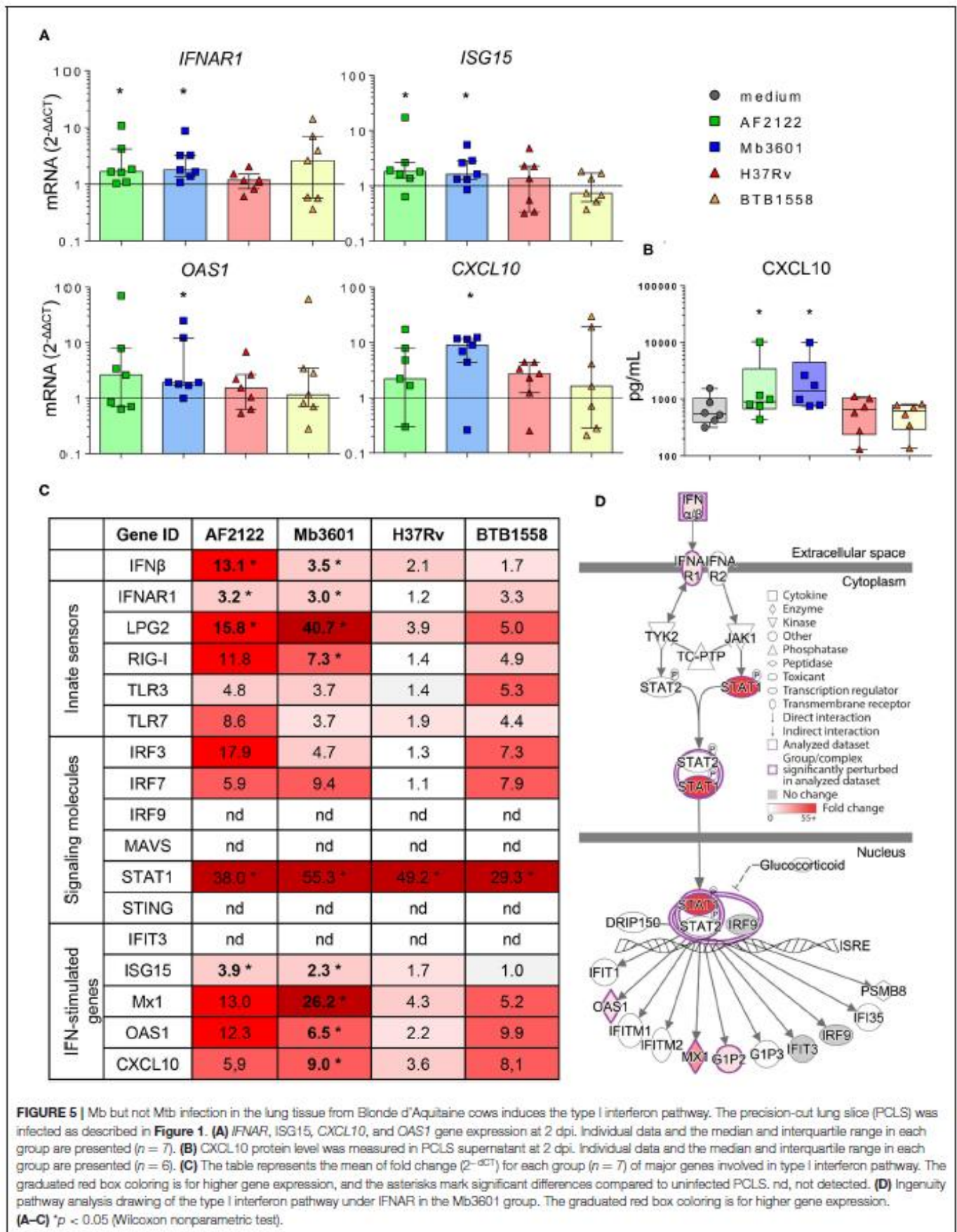
### The Type I Interferon Pathway Is Induced in the Bovine Lung by Infection With *M. bovis*, but Not *M. tuberculosis*

Because in humans and mouse models susceptibility to mycobacterial infection and disease progression is driven by type I IFN (35–37), we decided to compare the induction of this pathway by Mtb and Mb strains in bovine lung tissue. We measured the expression of different genes involved in the type I IFN pathway in Blonde d'Aquitaine PCLS infected by the four mycobacterial strains (Figure 5). The gene expression of both IFN $\beta$  and the IFNAR1 receptor was significantly increased after Mb but not Mtb infection (Figures 5A,C). Similarly, the major IFN-stimulated genes (ISG) *MX1*, *OAS1*, *ISG15*, and *CXCL10* were induced only after Mb infection (Figures 5A,C), and this difference was also detected at the protein level for *CXCL10* (Figures 5A,B). Therefore, we observed the induction of a number of genes of the type I IFN pathway, recapitulated in Figure 5D, after infection with Mb, but not Mtb, strains. Strikingly, strain Mb3601 was the highest inducer of this pathway in the lung from Blonde d'Aquitaine cows.

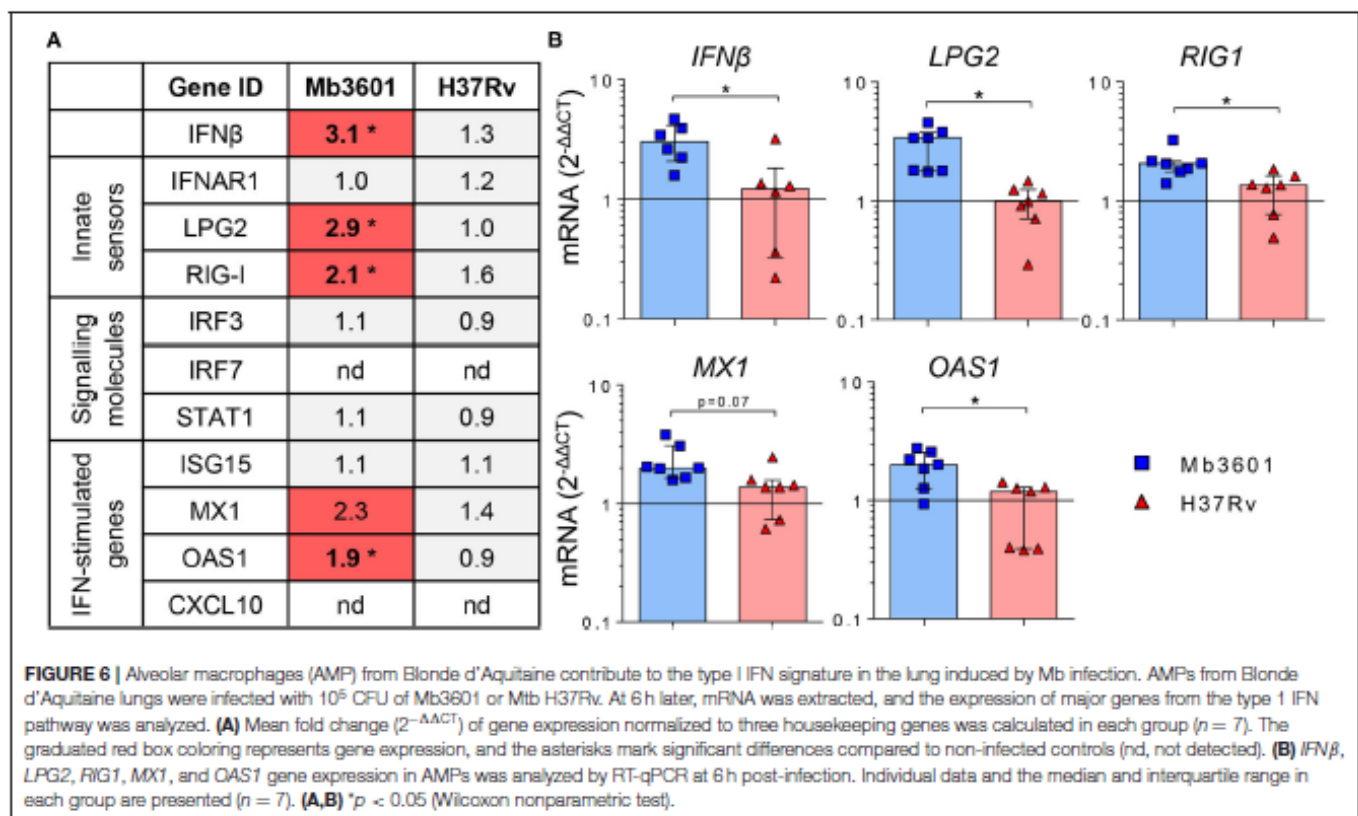
Because AMPs are the most prominent host cells interacting with Mb (8), which we also observed in PCLS (Figure 2), we next decided to decipher if AMPs contributed to the induction of the type I IFN pathway after Mb3601 or H37Rv infection. At 1 day after the infection of AMPs with these two strains, similar bacterial levels were recovered (data not shown). At 6 h post-infection, no cell cytotoxicity was observed, and we analyzed the expression of genes from the type I IFN pathway at this early time point. While we did not observe differences in *IFNAR1*, *IRF3*, *STAT1*, nor *ISG15* expression induced by the two strains (Figure 6A), *IFN $\beta$* , *LPG2*, *RIG1*, and *OAS1* were significantly induced after infection with Mb3601, but not H37Rv (Figure 6B). Regarding *MX1*, the same trend was observed, although statistical significance was not reached (Figure 6B,  $p = 0.07$ ).

Interestingly, while *CXCL10* was detected both at the mRNA and protein levels in PCLS infected with Mb (Figure 5), we did not detect the expression of this gene by AMPs in our analysis. These results altogether demonstrate that AMPs globally contribute to the type I IFN pathway in the lung after Mb infection, although other cells present in PCLS may also









specifically induce some genes, such as *CXCL10* or *IRF7*, for example (Figures 5C, 6A).

## DISCUSSION

The lung is the main organ targeted by Mb infection in cattle (38), and early interactions between the different lung cell types and the bacillus that govern the pathophysiology of the disease need to be better understood. In this study, we used PCLS for the first time to monitor the early bovine lung response to Mb infection and validated this model as a means to measure the local innate response at the protein and mRNA level. A main advantage of PCLS is conservation of the complex lung tissue both in structure and diversity of cell types. After infection with mycobacteria, the ciliary activity of bronchial cells was maintained. The AMP main function is to patrol the lung, crawling in and between alveoli; they sensed, chemotaxed, and phagocytosed debris or inhaled bacteria (39). We observed increased numbers of AMPs in alveoli where Mb was present, indicating AMP mobility inside the tissue. In chicken, PCLS allowed the observation of the movement of macrophages and phagocytosis (30). The AMP is well established as the main host cell for Mtb infection in humans (40) and Mb infection in cattle (41). Accordingly, in PCLS, we observed Mb inside AMPs in 20% of infected alveoli. We sometimes observed several bacilli inside one AMP. Although Mb is able to replicate inside this hostile cell, it is difficult to know if this observation was due to bacillary multiplication or the phagocytosis of several

bacilli. This issue would need live imaging of PCLS to follow the fate of fluorescent Mb, an approach which remains challenging under BSL3 conditions.

In uninfected PCLS, we observed generally one AMP for two to three alveoli [Supplementary Figure 4; in good correlation with the observations of Neupane et al. (39)]. After Mb infection, we observed several AMPs inside the same alveolus in 50% of cases. Moreover, when the alveoli contained more than four AMPs, they were in close contact. Multinucleated giant cells are formed by the fusion of several MPs and are a hallmark of TB pathophysiology. It has recently been demonstrated that, after infection of human or bovine blood-derived MPs by Mb or Mtb, only Mb was able to induce the formation of multinucleated cells (26). Although at 2 dpi we did not observe the formation of such cells in PCLS, it would be interesting to analyze if such events could be detected after longer infection periods. Goris et al. have maintained bovine PCLS during 1 week to study viral infections (27).

One other advantage of our model is the preserved diversity of lung cell composition. PCLS contain type I and II pneumocytes, endothelial cells, and bronchial cells (Supplementary Figure 4) and also produce key molecules like surfactant, which has an established role in Mtb uptake (42). Mtb is also capable of invading type II alveolar epithelial cells (23) that play important roles in host defense (20–22). In our study, we did not observe intraepithelial Mb, but specific labeling of bovine epithelial cells would be required to investigate interactions between bovine lung pneumocytes and Mb in more detail. However, as we have

observed that infected AMPs were in close contact with epithelial cells in PCLS, this model will allow a more refined analysis of the crosstalk between AMPs and pneumocytes during Mb infection (24).

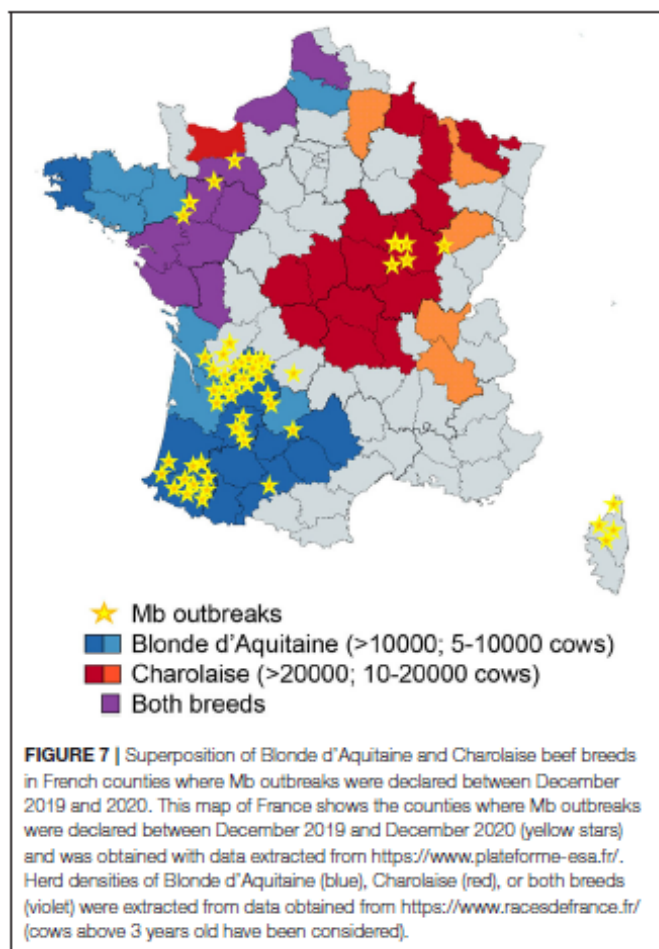
One limitation of the PCLS model is the lack of recruitment of immune cells from circulating blood. During mycobacterial infection, in response to local signals, a variety of immune cells are recruited to the infection site to form the mature granuloma that constrains bacillary multiplication. How this response is orchestrated at the level of the lung tissue in cattle remains poorly established. Neutrophils, together with other innate cells, such as macrophages,  $\gamma\delta$ -T lymphocytes, and natural killer cells, were recently identified as key immune cells in the early containment of infection (43) and development of early lesions (44). Moreover, humans regularly exposed to Mtb or cattle exposed to Mb do not always develop signs of infection, i.e., remain negative in IFN $\gamma$ -release assay or skin testing. In humans, such resistance to infection through the successful elimination of bacilli could be mediated by neutrophils (45). Similarly, in cattle experimentally infected with Mb, some contact animals resist infection, while others develop lesions due to productive infection (46). It is possible that neutrophils could also play an important role in the early elimination of Mb in cattle (43). Immune signals involved in the early recruitment of neutrophils to the lung after the entry of Mb need to be better understood in cattle. It is known that epithelial cells secrete, among other cytokines and chemokines, MIP1 and CXCL8 that attract MPs and neutrophils to the site of infection. Interestingly, we measured important differences in the production of such mediators by PCLS in response to different strains of mycobacteria that could be linked to variable virulence. Although one cattle type II pneumocyte cell line has been described (47), such transformed cells are less physiologically relevant than primary cells. Recently, immortalized type II cells were co-cultured with endothelial cells as a model of the bovine alveolus to study mycobacterial interactions with BCG. In this study, the authors detected the production of IL-8, TNF $\alpha$ , IL-22, and IL-17a. One limitation of this model was epithelial cell death, which occurred shortly after infection (48). As a physiological model, PCLS could help in understanding the early orchestration of the local inflammatory response in the lung in response to mycobacterial infection.

Resistance to bTB is linked to the host genetics. Zebu breeds (*Bos indicus*) are more resistant to bTB disease than *Bos taurus*-derived breeds (49). Our results with PCLS, as a physiological model of the early lung response to infection, demonstrated striking differences between Blonde d'Aquitaine and Charolaise, emphasizing the importance of host genetics in response to Mb. It is not known whether the stronger inflammatory response of the Blonde d'Aquitaine tissue is associated with a greater sensitivity or resistance to Mb infection. While robust immunological responses are associated with an increased pathology at the level of the animal (31), at the cellular level, blood-derived MPs from animals with greater resistance to bTB (and that kill BCG more efficiently than cells from susceptible animals) produce higher levels of the pro-inflammatory mediators iNOS, IL-1 $\beta$ , TNF $\alpha$ , MIP1, and MIP3 (25). Although genetic selection of cattle would greatly complement bTB management and

surveillance programs to control and ultimately eradicate the disease, especially in countries with the highest burden (50, 51), biomarkers to evaluate the resistance or susceptibility of cattle to Mb infection are critically missing. Some genomic regions and candidate genes have been identified in Holstein-Friesian cows, the most common dairy breed (52), and not surprisingly, these candidates are often involved in inflammation. A genomic region on chromosome 23, containing genes involved in the TNF $\alpha$ /NF $\kappa$ -B signaling pathway, was strongly associated with host susceptibility to bTB infection (53). However, large within-breed analyses of Charolaise, Limousine, and Holstein-Friesian cattle identified 38 SNPs and 64 QTL regions associated with bTB susceptibility to infection (54). The genotyping of 1966 Holstein-Friesian dairy cows that were positive by skin test and either did or did not harbor visible bTB lesions, together with their skin test negative matched controls, led to the conclusion that these variable phenotypes following Mb exposure were governed by distinct and overlapping genetic variants (55). Thus, variation in the pathology of Mb seems to be controlled by a large number of loci and a combination of small effects. Similar conclusions were drawn from the genetic studies of human tuberculosis (56). In areas where Mb is highly prevalent, recurrent exposure to Mb may also imprint the bovine genome, and epigenetics could also contribute to the immune response in certain breeds. In France, the Nouvelle Aquitaine region accounted for 80% of Mb outbreaks last year. Interestingly, Blonde d'Aquitaine breed is very abundant in this area (Figure 7). Together with Limousine, another very abundant beef breed in this region, they contribute to most bTB outbreaks in Nouvelle Aquitaine (bovine tuberculosis national reference laboratory communication). In the future, comparisons between Blonde d'Aquitaine and Limousine would be interesting. In our study, Blonde d'Aquitaine or Charolaise cows were sampled from eight different French departments, none with recurrent Mb outbreaks, rendering previous exposure to Mb unlikely. Moreover, the breeding management was similar for the two breeds, as far as we could ascertain, suggesting that exposure to environment and possible wildlife sources would be comparable. We nevertheless observed striking differences in the early lung response to Mb infection between these two breeds, pointing to the possible control of Mb infection at the genetic or epigenetic level. Whether some cattle breeds are more susceptible to bTB than others remains an open question that deserves future studies with more consequent animal sampling. We furthermore believe that the PCLS model could greatly contribute to unraveling the role of tissue-level protective responses that would, in turn, reveal important biomarkers.

In addition to the cattle breed, our study pointed toward differences in the host response to distinct mycobacterial strains. The Mb strains were better inducers of a lung immune response than Mtb in cattle, which is in agreement with a previous work showing that Mtb H37Rv was attenuated *in vivo* in cattle compared to Mb AF2122 (13). *In vitro* studies with bovine AMPs infected with AF2122 or H37Rv revealed differences in the innate cytokine profiles: the CCL4, IL-1 $\beta$ , IL-6, and TNF $\alpha$  levels were more elevated in response to AF2122 than H37Rv (8), which is in agreement with our data. Interestingly, Mb3601,





a representative strain of a highly successful genetic cluster that circulates both in cattle and wildlife in France (16), induced an inflammatory signature in the lung more efficiently than Mb AF2122. Whether this correlates with differences in Mb virulence in cattle or other mammals remains to be investigated; but, if this were the case, the PCLS model would be a practical tool to study and compare the virulence of Mb field strains compared to the *in vivo* experimental infection of cattle. Contrary to Mtb which is mostly restricted to humans, Mb is adapted to sustain across a large host range through repeated cycles of infection and transmission (57, 58). This remarkable trait is due to pathogen molecular genetic changes (59) that allow adapted bacilli to manipulate the host immune response to establish infection and disease and ultimately transmit infection to new, susceptible hosts (60, 61). We observed a weaker inflammation in the bovine lung after infection with Mtb compared to Mb, and it will be interesting to compare the ability of Mtb and Mb to induce inflammation in human PCLS obtained post-surgery. This latter comparative analysis could give clues on the links between lung innate inflammatory responses and host adaptation during TB.

Our most striking observation was the Mb-restricted induction of the type I IFN pathway in the bovine lung. This is in agreement with previous studies in bovine AMPs where cytosolic

DNA-sensing pathways, in particular, RIG-I, were activated after 48 h of infection by Mb AF2122, but not Mtb H37Rv (32). In agreement with our data, these authors also demonstrated an induction of the RIG-I signaling pathway by Mb in AMPs (62). Therefore, AMPs contribute to type I IFN signaling in the lung. However, we also noticed differences between PCLS and AMPs in the induction of the IFN signature by Mb: for example, CXCL10 was detected in PCLS, but not in AMPs, in our study, which may be due to the time point used (63). However, it is also possible that other cells involved in crosstalk with AMPs contributed to CXCL10 production in response to Mb infection. Since CXCL10 has been proposed as a diagnostic biomarker of Mb infection in cattle (64), it will be interesting to better understand how this key mediator is regulated. Type I interferon favors Mb survival, and its induction may be a good manipulation strategy for the maintenance of infection. This manipulation mechanism, deciphered *in vitro* in murine bone marrow monocyte-derived MPs, involves the triggering of autophagy by cytosolic Mb DNA, in turn inducing IFN $\beta$  production. Autophagy antagonizes inflammasome activation to the benefit of Mb survival (65, 66). In C57BL/6 mice treated with IFNAR1 blocking Ab and infected with Mb, the recruitment of neutrophils was reduced, but the pro-inflammatory profile of MPs was increased, leading to a reduced bacillary burden (67). No impact on T-cells was observed in this *in vivo* model, revealing a role of type I IFN signaling during the innate phase of the host response to infection. Therefore, Mb exploits type I IFN signaling in many ways, and this pathway seems an important avenue to better understand Mb virulence. The PCLS model will greatly help to better dissect out this pathway in the lung during bTB. This could lead to new biomarkers to help genomic selection programs for cattle that are more resistant to bTB as well as new immunostimulation strategies counteracting the type I IFN pathway. This new knowledge will ultimately improve bTB control, a goal which is so greatly needed at the global level (68).

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

Ethical review and approval was not required for the animal study because We only used post-mortem sampling at commercial abattoir.

## AUTHOR CONTRIBUTIONS

AR designed and did most of the experiments, obtained funding, analyzed the data, prepared all the figures, and wrote the manuscript. FC performed experiments and prepared the inocula for experimental infections under BSL3 conditions. AC cultured AMPs and performed ELISA and q-RT-PCR. ED-D helped in PCLS experiments and revised the figures. MB provided the



Mb3601 strain and revised the manuscript. AA provided the strain Mtb BTB1558. JB improved the RNA extraction protocol. DD and QM performed multiple experiments, and revised the manuscript. FA provided Ab and critically reviewed the imaging data. PG helped with transcriptomic analysis and revised the manuscript. SG obtained funding, designed the experiments, and revised the manuscript. NW obtained funding, supervised all aspects of the work, critically analyzed the data, and wrote the manuscript. All the authors read and approved the manuscript before publication.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fvets.2021.696525/full#supplementary-material>

**Supplementary Table 1** | Sequences of primers used in this study. The primers were designed, using Geneious software, in intron-spanning regions when possible. The annealing temperature was set at 60°C. Housekeeping genes used as the reference to calculate  $\Delta\text{CT}$  are indicated in the gray boxes.

**Supplementary Figure 1** | Age and geographical origin of the cows used in the study. The Charolaise and Blonde d'Aquitaine cows used were between 3 and 11

years old and came from eight different French departments. Two Blonde d'Aquitaine cows came from the same farm in Indre et Loire, and three Charolaise cows came from the same farm in Sarthe. All the other animals are from distinct farms. The data represent the age of individual animals and the median and interquartile range.

**Supplementary Figure 2** | Transcriptomic signature after infection with different doses of mycobacteria. Bovine precision-cut lung slices were obtained as described in **Figure 1** and infected with  $10^5$ ,  $5 \times 10^5$ ,  $10^6$ , or  $5 \times 10^6$  colony-forming units. The RNA was extracted 2 days post-infection, and *SDC4*, *CXCL1*, *HIF1*, and *OAS1* gene expressions were assessed with the Fluidigm Biomark. Individual data and the mean and standard deviation in each group are presented ( $n = 3$  Charolaise). The dotted line represents the level of expression in the uninfected group.

**Supplementary Figure 3** | Cytokines/chemokines in precision-cut lung slice (PCLS) supernatants. The protein levels were measured in PCLS supernatant at 2 days post-infection with Multiplex. Individual data and the median and interquartile range in each group are presented ( $n = 6$ ). \* $p < 0.05$  (Wilcoxon nonparametric test).

**Supplementary Figure 4** | Structure of the bovine precision-cut lung slices (PCLS) under a light microscope. The PCLS were observed under a light microscope (enlargement  $\times 40$  to  $\times 200$ ). The PCLS contain numerous alveoli and between one to three bronchioles, with thick and wavy epithelium that can be easily recognized (black asterisk, two views from the same area under two enlargements). Thin blood vessels (red dotted lines) were localized next to the bronchioles and diffused between the alveoli. No blood cells remained inside the endothelium (the cows were bled out at the abattoir). Alveolar macrophages can be seen inside the alveoli (black arrows).

**Supplementary Figure 5** | Localization of Mb3601-GFP in bovine precision-cut lung slices (PCLS). The PCLS were fixed at 2 days post-infection with  $10^5$  colony-forming units of Mb3601-GFP recombinant strain and labeled with anti-pancytokeratine and anti-MHCII antibodies, which, respectively, revealed anti-pancytokeratine and Alexa 555 conjugated secondary Ab. The PCLS were transferred on cover slides and mounted with Fluoromount-G™ mounting medium containing DAPI. **(A)** The 3D images were analyzed with Leica LAS software. Z-stack imaging was performed at  $\times 63$  enlargement with a confocal microscope (10–15  $\mu\text{m}$  in thickness, step size of 0.5–1  $\mu\text{m}$ ). Dotted white lines are drawn on the alveoli structure. **(B,C)** Crosshead sections illustrating Mb3601 inside **(B)** or near **(C)** an alveolar macrophage. X and Y projections are seen on the bottom and to the right of the picture; the intracellular localization of Mb3601-GFP is indicated by color merging (green + red = yellow). The results from one representative animal are shown (a total of  $n = 4$  animals were analyzed).

**Supplementary Video 1** | Internalization of Mb3601 in alveolar macrophages after precision-cut lung slice (PCLS) *ex vivo* infection. The PCLS was fixed at 2 dpi with  $10^5$  colony-forming units of Mb3601-GFP recombinant strain and labeled with anti-pancytokeratine and anti-MHCII antibodies, which, respectively, revealed anti-pancytokeratine and Alexa 555 conjugated secondary Ab. The PCLS was transferred on cover slides and mounted with Fluoromount-G™ mounting medium containing DAPI. Z-stack imaging was performed at  $\times 63$  enlargement with a confocal microscope. The 3D images were analyzed with Leica LAS software.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past collaboration with one of the authors SG.

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## 9. Summary in French - Synthèse en français

La synthèse ci-après résume mes activités de recherche antérieures et post-recrutement ; mes activités d'encadrements et enseignements/évaluation. Je n'ai pas traduit le préambule, mon CV ni l'annexe qui liste de façon exhaustive mes publications, présentations et posters.

### SOMMAIRE

<b>A. Résumé des travaux de recherche antérieurs à mon recrutement .....</b>	<b>p154</b>
<b>B. Résumé de mes travaux depuis mon recrutement .....</b>	<b>p156</b>
○ Eléments de contexte : enjeux sociaux-économiques et scientifiques de la bTB.....	p157
○ Etude des réponses pulmonaires précoces à l'infection.....	p159
○ Première caractérisation de deux sous-populations de neutrophiles bovins.....	p160
○ Eléments de contexte : enjeux sociaux-économiques & scientifiques des mammites	p161
○ Les rôles des neutrophiles bovins lors d'une infection mammaire chez la vache .....	p161
<b>C. Tableau résumant mes publications.....</b>	<b>p163</b>
<b>D. Tableau résumant mes projets financés.....</b>	<b>p164</b>
<b>E. Expériences d'encadrement.....</b>	<b>p165</b>
<b>F. Expériences d'enseignement .....</b>	<b>p167</b>
<b>G. Perspectives .....</b>	<b>p169</b>
○ Comprendre le rôle des sous-populations de neutrophiles dans la physiopathologie de la bTB (ANR JCJC Neutro_bTB 2022-2025).....	p169
○ Rôle des neutrophiles dans d'autres pathologies (mammites).....	p172
○ Modèle PCLS : une bonne visibilité qui attire des collaborations .....	p174

### A. Résumé des travaux de recherche antérieurs à mon recrutement

A la fin de mon master, j'ai obtenu un Contrat Jeune Scientifique (CJS) pour financer 5 années de recherche à l'INRAE (thèse+post-doc). J'ai effectué ma thèse sous la direction du Dr. S. Riffault (UR892 VIM, INRA de Jouy-en-Josas). Mon travail a consisté à étudier les spécificités de l'immunologie du nouveau-né dans trois contextes différents : à l'homéostasie, lors d'une infection par le Virus Respiratoire Syncytial (agent de la bronchiolite) et dans le cadre d'une vaccination contre ce virus. J'ai utilisé les modèles murins pour décrypter rapidement des

mécanismes immuno-pathologiques et j'ai travaillé dans des espèces d'intérêt agronomique, bovin (mobilité à l'université vétérinaire d'Hanovre, Allemagne) et ovin. J'ai mis en évidence plusieurs déficits de l'immunité néonatale (interféron de type I, cellules dendritiques) contribuant à la plus grande sensibilité des nouveau-nés au virus. J'ai consolidé mon portfolio de compétences en immunologie et acquis des compétences pointues dans plusieurs domaines notamment en biologie moléculaire (microarray). J'ai appris à gérer le travail dans des conditions de terrain (ferme expérimentale pour les ovins, abattoir industriel pour les bovins) et à m'organiser autour d'expérimentations souvent lourdes (chirurgie pour les agneaux prématurés). Mes résultats de doctorat ont été valorisés par trois articles scientifiques en 1<sup>er</sup> auteur, un article collaboratif en 5<sup>ème</sup> auteur et la participation à de nombreux congrès européens et internationaux.

A la fin de ma thèse, j'ai décidé de m'intéresser à l'impact du microbiote pulmonaire sur l'immunité pulmonaire à la naissance. J'ai rédigé mon projet de post-doctorat dans le cadre de mon CJS. En mars 2013, j'ai intégré l'équipe ProbiHôte, spécialiste de l'étude du dialogue entre bactéries probiotiques et commensales avec l'Hôte (UMR1319 Micalis, INRA Jouy-en-Josas) sous la direction du Dr. M. Thomas. J'ai choisi cette équipe pour ses compétences en physiologie et microbiologie digestives ; j'y ai apporté mes connaissances en santé respiratoire et immunologie. Pour évaluer l'impact du microbiote lors d'une pathologie respiratoire, j'ai souhaité travailler dans un autre modèle que celui de ma thèse, et j'ai obtenu un financement Agreenskills pour effectuer une mobilité de 6 mois dans le laboratoire des Pr. B. Lambrecht et Pr. H. Hammad à Gent, dans lequel j'ai étudié les modulations du microbiote pulmonaire dans un modèle d'asthme allergique, induit chez la souris à partir d'un broyat d'acariens. Lors de mes études sur l'impact sur la santé de l'implantation du microbiote sur la maturation post-natale de l'épithélium et du système immunitaire respiratoire, j'ai identifié deux souches bactériennes isolées de poumon de souris avec des effets antagonistes : l'une exacerbant l'inflammation post-asthme, l'autre induisant une protection partielle. Mes résultats de post-doctorat ont été valorisés par le dépôt d'un brevet, un article scientifique en 1<sup>er</sup> auteur, la publication d'un article collaboratif en 2<sup>ème</sup> auteur et la participation à de nombreux congrès européens et internationaux.

Lors de ce stage postdoctoral, j'ai montré ma capacité à conduire un projet de recherche depuis sa conception jusqu'à sa réalisation et sa valorisation. J'ai su m'adapter à de nouveaux environnements de travail, une unité de microbiologie qui développe des approches novatrices en bactériologie et physiologie, et un laboratoire d'immunologie renommé à l'étranger. J'ai diversifié mes compétences avec des expériences en bactériologie et la manipulation d'animaux axéniques. J'ai recherché et obtenu mes propres sources de financements, partie importante du travail d'un chercheur. Par ailleurs, tout en poursuivant mes travaux de recherche, je suis intervenue dans différents enseignements (j'ai été co-responsable d'un module d'immunologie moléculaire pour des étudiants de Master1 par

exemple), et j'ai encadré plusieurs étudiants. J'ai montré mon aptitude pédagogique et mon intérêt pour la formation et la transmission des connaissances.

## B. Résumé de mes travaux depuis mon recrutement

Après un concours qui s'est ouvert dans l'unité Infectiologie et Santé Publique d'INRAE Centre Val de Loire, je suis arrivée dans l'unité Infectiologie et Santé Publique en décembre 2015, en tant que chargée de recherche, pour étudier la physiopathologie de la tuberculose bovine (bTB). En 2018, l'équipe travaillant sur les mycobactéries a fusionné avec une autre équipe étudiant les mammites bovines, ensemble elles forment ma nouvelle équipe, IBIR, pour Infections Bactériennes et Immunité des Ruminants. Bien que mes projets de recherche soient principalement axés sur la bTB, je participe activement aux projets mammites sous l'angle des neutrophiles.

En tant qu'immunologiste bovine travaillant à INRAE, mes ambitions sont de :

- Développer des outils immunologiques et moléculaires pour les bovins
- Comprendre les facteurs innés qui contribuent au contrôle de *Mycobacterium bovis* (Mb, l'agent de la bTB) dans le poumon bovin
- Identifier les corrélats de protection contre l'infection à Mb

Depuis 2018, j'ai un fort intérêt pour les neutrophiles. Le rôle clé des neutrophiles dans la tuberculose humaine est établi. Le double rôle des neutrophiles inflammatoires et des populations suppressives de cellules suppressives myéloïdes granulocytaires (MDSC) - qui sont phénotypiquement proches des neutrophiles - sur le devenir de la tuberculose a récemment été décrit chez des patients tuberculeux humains et des modèles murins. Les informations sur le rôle des neutrophiles bovins au cours de la bTB sont rares. Pour les projets liés aux neutrophiles que je mène, je vise à :

- Mieux caractériser les neutrophiles chez les bovins
- Comprendre le rôle des sous-populations de neutrophiles dans la physiopathologie de la bTB

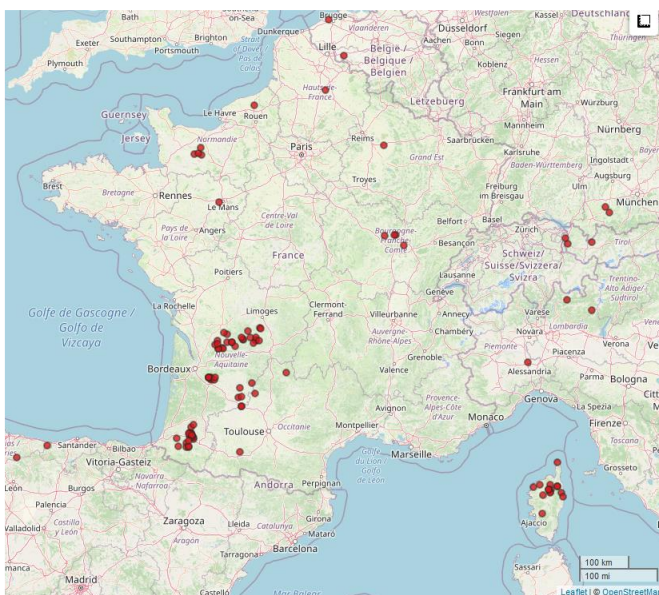
Les neutrophiles sont essentiels pour le contrôle des mammites, et dans l'équipe IBIR, nous pensons que l'amélioration des connaissances sur ces acteurs majeurs conduira à affiner les programmes de diagnostic ou de sélection génomique bovine. Pour les projets mammites, j'ambitionne de :



- Caractériser le rôle des sous-populations de neutrophiles dans le contexte des mammites cliniques et subcliniques

### Éléments de contexte : enjeux sociaux-économiques et scientifiques de la bTB

Au niveau mondial, les pertes économiques pour le monde agricole dues à la bTB sont estimées à 3 milliards de dollars (Schiller et al., 2010). La bTB est une zoonose qui pose de graves problèmes en santé animale et humaine dans le monde (Olea-Popelka et al., 2017)<sup>3</sup>.



Dans les pays où la surveillance de la bTB est bien établie, comme en France, les symptômes de la maladie sont très rarement observés car les animaux sont éliminés avant d'entrer en phase clinique. Un dépistage positif de la maladie, par un test de sensibilité à la tuberculine et/ou de production d'interféron gamma (IFN $\gamma$ ), entraîne l'abattage de l'animal voire du troupeau entier. Les animaux peuvent être excréteurs et contaminer leurs congénères sans présenter de signes cliniques évidents et le diagnostic n'est

pas efficace à 100% pour dépister les animaux infectés. Selon les chiffres donnés par le ministère de l'agriculture, le programme de contrôle pour maintenir la prévalence de bTB au-dessous des 0,1% des troupeaux coûte environ 20 millions d'euros par an (75% pour assainissement et indemnisation des éleveurs, 1er poste de dépense du ministère de l'agriculture pour le contrôle d'un agent pathogène unique). Cependant, malgré ce programme coûteux, la recrudescence de cas depuis 2010 menace le statut de la France officiellement indemne de bTB. Tous les ans, Mb est identifié au sein de foyers résiduels (Dordogne, Côte d'or, Camargue, Sud-Ouest, carte ci-contre, <https://shiny-public.anses.fr/shiny-vsi/>). Pour étudier ce phénomène, INRAE et l'ANSES se sont associés pour étudier la répartition et la diversité des souches de Mb, et ont ainsi pu démontrer que cette régionalisation traduisait un succès épidémiologique de certaines souches modernes, et non un phénomène de réémergence. La persistance de ces foyers résulte également d'une implantation et d'une diffusion dans la faune sauvage, qui jouerait un rôle de réservoir (Hauer et al., 2015). L'organisation de l'élevage bovin favorise la prévalence de certains génotypes, mais la contribution d'une plus grande virulence de certaines souches est encore en débat. On sait aussi que certains génotypes sont dominants et capables de circuler dans un système multi-hôtes, mais on ignore si cela se traduit par des phénotypes différents.

<sup>3</sup> <https://www.woah.org/app/uploads/2021/03/roadmap-zoonotic-tb.pdf>

Du côté de l'hôte, la résistance génétique à la bTB a été établie pour des races de vaches exotiques comme la Sahiwal (*Bos indicus*) vs la Holstein-Friesian (HF, *Bos taurus*) (Ameni et al., 2007). La modulation de l'immunité, déterminée au niveau génétique, pour contrôler l'infection Mb reste un domaine peu exploré. L'identification de biomarqueurs immunologiques de sensibilité ou résistance à la maladie pourrait impacter les programmes de sélection génétique de bovins actuellement basés essentiellement sur des critères de production et reproduction. Chez l'homme infecté par *Mycobacterium tuberculosis* (Mtb, du même complexe génétique que Mb), de nombreux travaux montrent un contrôle multigénique de la sensibilité/résistance à la tuberculose/maladie (Casanova and Abel, 2002). Par contre, la résistance à l'infection est encore incertaine car difficilement traçable parmi les cas contacts. En effet, la majorité des individus (animaux ou Hommes) peut contrôler et éliminer l'infection sans signature immunitaire spécifique, ce qui traduit le rôle primordial de l'immunité innée (Cassidy and Martineau, 2014). Parmi les cellules de l'immunité innée, les macrophages alvéolaires (MA) sont les premières cibles de Mb. Activés correctement, les MA sont capables de détruire les bactéries phagocytées par la voie lysosomale et par la sécrétion de molécules bactéricides comme les « Reactive Oxygen Species » (ROS). Cependant, certains bacilles sont capables d'échapper à la lyse et de survivre dans les MA, faisant de ces cellules des foyers de ré-infection ou de dissémination (Pieters, 2008). Ces zones inflammatoires chroniques évoluent en granulomes (structures complexes et organisées, composées de plusieurs types cellulaires appartenant à l'immunité innée et adaptative), qui ont pour vocation de contenir les bacilles à défaut de les éliminer. Le rôle des macrophages pour le contrôle de Mb chez le bovin a été étudié principalement à partir de cellules dérivées de précurseurs sanguins (Castillo-Velázquez et al., 2013; Qureshi et al., 1996), et quelques études récentes avec des MA ont démontré leur rôle majeur pour l'initiation de la réponse inflammatoire suite à une infection *in vitro* et le détournement de leur machinerie cellulaire par Mb pour sa survie (Malone et al., 2018; Nalpas et al., 2015; Vegh et al., 2015).

Le neutrophile est l'autre cellule majeure de l'immunité innée. Longtemps considérée comme uniquement phagocytaire, cette vision s'est modifiée grâce à la description de sous populations granulocytaires ayant des propriétés régulatrices. Les neutrophiles sont toutefois des armes à double tranchant car en l'absence de contrôle, leur réponse peut conduire à une dégradation des tissus (Dallenga et al., 2017). Dans le cas de la tuberculose, les neutrophiles sont recrutés dans le poumon en plusieurs vagues (Lombard et al., 2016), ils contribuent à l'élimination des mycobactéries et à la formation du granulome mais sont aussi les principaux responsables de l'inflammation délétère (Dallenga et al., 2017; Dallenga and Schaible, 2016). Leur rôle dans le cadre d'une infection par Mb est très peu documenté, même chez la souris. Chez le bovin, les neutrophiles représentent 30% des leucocytes circulants. Cependant, peu de données sont disponibles sur ces cellules, et uniquement quelques publications y font référence dans le cadre d'une infection par Mb. En 1998, Cassidy et al. ont observé les stades précoces des lésions après une infection expérimentale chez des

veaux, et conclu au rôle clé des neutrophiles pour la formation des granulomes (Cassidy et al., 1998). Une étude publiée en 2013 décrit que la phagocytose de Mb par des neutrophiles déclenche la production de cytokines inflammatoires mais que les cellules sont incapables d'éliminer la bactérie (Wang et al., 2013b). Les mêmes auteurs ont observé une modulation des gènes signatures des neutrophiles, comme celui de la myéloperoxydase et PTX3, chez des bovins infectés par Mb (Wang et al., 2013a). Enfin une autre étude chez le bovin suggère un rôle délétère des neutrophiles à travers une production d'IL-17, qui induirait une forte réponse pro-inflammatoire dans les lésions pulmonaires, ainsi que la production d'arginase qui pourraient diminuer l'élimination de Mb car elle entrerait en compétition avec iNOS pour l'utilisation de la L-arginine (Shu et al., 2014). Mb exploite les voies de l'IFN de type I pour sa survie dans les macrophages (Chunfa et al., 2017; Malone et al., 2018; Wang et al., 2019). On ne sait pas encore si les neutrophiles bovins contribuent à la signature IFN de type I, comme cela a été démontré chez des patients tuberculeux actifs (Berry et al., 2010).

### Etude des réponses pulmonaires précoces à l'infection

A mon arrivée dans mon unité, j'ai implémenté le modèle d'explants pulmonaires appelé Precision cut lung slices (PCLS) que j'ai appris lors de mon séjour à Hanovre lors de ma thèse.

Je voulais étudier la réponse innée bovine suite à une infection par Mb dans un environnement pulmonaire préservé pour permettre aux cellules pulmonaires résidentes d'interagir entre elles et avec les bacilles. Les PCLS sont un modèle expérimental dans lequel l'architecture tissulaire est conservée, et les types de cellules pulmonaires résidentes sont préservées et restent vivantes pendant au moins une semaine (Goris et al., 2009).

En collaboration avec le Pr. S. Gordon du UCD, j'ai décidé de comparer les souches Mb et Mtb, (projet PHC ONE-TB, co-coordonné par Pr. Gordon et moi-même). Étant donné que le patrimoine génétique de l'hôte a également un impact sur le développement de la bTB (Allen, 2017), j'ai décidé de comparer les réponses des PCLS de deux races à viande prévalentes en France, Charolaise et Blonde d'Aquitaine, et j'ai mené une caractérisation approfondie des réponses pulmonaires à Mb et Mtb lors d'une infection *ex vivo*. (Remot et al., 2021)

Nous avons observé des différences importantes entre les races bovines (niveau des réponses plus élevé chez la Blonde d'Aquitaine par rapport à la Charolaise) et les souches mycobactériennes (signature inflammatoire variable en fonction des souches, activation des voies de l'IFN de type I avec Mb et pas Mtb). Ce modèle pourrait aider à identifier des biomarqueurs de résistance *versus* sensibilité à la bTB. Ces informations sont encore indispensables pour les programmes de sélection génétique bovine et contribueraient grandement à l'effort mondial d'éradication de la bTB.



## Première caractérisation de deux sous-populations de neutrophiles bovins

Les neutrophiles sont des cellules immunitaires clés. Longtemps considérés comme des « kamikazes », ces phagocytes, acteurs de l'immunité innée, se sont érigés en acteurs majeurs de la réponse immunitaire avec la découverte de nouvelles fonctions. En plus de leurs propriétés antimicrobiennes uniques, ils participent pleinement à la formation de l'immunité adaptative par dialogue avec d'autres cellules et à la suppression de la prolifération et de l'activité des lymphocytes T (Müller et al., 2009). Ils peuvent également migrer vers les ganglions lymphatiques drainants, comme les DC ou les monocytes inflammatoires, et y transporter des micro-organismes vivants (Abadie et al., 2005). Ce large éventail de phénotypes et de fonctions a récemment été lié à des populations discrètes de neutrophiles caractérisées par des analyses à l'échelle de la cellule isolée (Lyadova, 2017). Ces nouveaux rôles sont désormais documentés pour l'homme et la souris. Forts des résultats sur le modèle murin obtenus au laboratoire par le Dr Doz-Deblauwe, nous avons récemment procédé à une caractérisation approfondie des neutrophiles circulant dans le sang des bovins à l'homéostasie. Ce travail a été initié avec le projet BoNeuro (2018-2019), financé par l'Institut Carnot France Future Elevage, que j'ai coordonné.

En copiant - au plus proche possible - le panel de cytométrie en flux et la stratégie de gating mis au point chez la souris par ma collègue (Dr Doz-Deblauwe), nous avons identifié une nouvelle sous population de neutrophiles régulateurs exprimant le complexe majeur d'histocompatibilité de type II (MHC-II), qui circulent dans le sang des bovins (Rambault et al., 2021a). Ces neutrophiles sont morphologiquement indiscernables des neutrophiles classiques négatifs pour le MHC-II. Cependant, les neutrophiles MHC-IIpos et MHC-IIneg présentent des profils transcriptomiques distincts. Alors que les neutrophiles MHC-IIneg et MHC-IIpos triés possèdent une activité de phagocytose ou de destruction bactérienne similaire, la population MHC-IIpos produit un niveau plus élevé de ROS et est capable de supprimer la prolifération des lymphocytes T.

En résumé, nous avons caractérisé deux sous-populations de neutrophiles et découvert - pour la première fois chez les bovins - une nouvelle population de neutrophiles régulateurs qui exprime MHC-II et possède une activité suppressive sur les lymphocytes T (Rambault et al., 2021a). Nos résultats mettent en avant les neutrophiles comme de véritables partenaires de la réponse immunitaire adaptative, y compris chez les espèces domestiques. Ils ouvrent la voie à la découverte de nouveaux biomarqueurs et d'interventions thérapeutiques pour mieux contrôler les maladies bovines.

Nous pensons que la diversité des phénotypes et des fonctions des neutrophiles doit être étudiée plus en détail et nous proposons de revisiter la description classique des neutrophiles en tant que population de cellule homogène chez les bovins. La fonction des neutrophiles MHC-IIpos au cours des infections reste inconnue et ce sujet est mon objectif actuel et mes perspectives pour les années à venir. Les interactions entre neutrophiles et Mb sont

abordées dans la partie perspectives, puisque ce projet vient d'être lancé (ANR JCJC Neutro\_bTB 2022-2025).

### **Eléments de contexte : enjeux sociaux-économiques et scientifiques des mammites**

La production laitière tient une place de choix dans l'agriculture et l'industrie agroalimentaire française. Son poids économique majeur de 30 milliards d'euros en fait la deuxième industrie agroalimentaire après la filière viande et génère un solde commercial positif de 3,2 milliards d'euros<sup>4</sup>. Les mammites ont un impact important sur l'économie de la filière, d'une part en raison de leur forte prévalence (20% des vaches d'un troupeau déclarent une mammite clinique au cours d'une lactation, et presque 30% des quartiers des vaches d'un troupeau sont atteints d'une mammite subclinique à un instant donné. De par leur nature inflammatoire et la douleur qu'elles engendrent, les mammites sont également responsables d'une altération du bien-être des animaux et de leur comportement (Gonçalves et al., 2017; Halasa et al., 2007). Actuellement, le traitement des mammites repose majoritairement sur l'utilisation d'antibiotiques : les mammites sont ainsi la première indication pour l'utilisation d'antibiotiques en élevage bovin (Halasa et al., 2007), ce qui n'est pas sans poser des problèmes notamment de résidus dans l'environnement et d'émergence de souches résistantes. Ainsi, la prévention des mammites est une approche qui permettrait d'améliorer l'économie de la filière laitière, le bien-être des animaux et de réduire l'utilisation d'antibiotiques en élevage. L'amélioration des compétences immunitaires des vaches laitières par la sélection génétique est une approche prometteuse pour réduire l'impact des mammites en élevage laitier (Lefebvre et al., 2020). De fait, pour la race Prim'Holstein, une part significative de l'index Santé de la mamelle (STMA), basé notamment sur les comptages des cellules somatiques (CCS) du lait, entre dans le calcul de l'index ISU 2012. Lors d'une infection mammaire, l'augmentation du CCS est majoritairement due à l'afflux de neutrophiles. Ces derniers ont un rôle primordial dans l'élimination des agents pathogènes, notamment lors d'infection à *Escherichia coli* : ainsi, une déplétion des neutrophiles ou un recrutement tardif entraîne une sévérité accrue des infections mammaires (Koess and Hamann, 2008; Rainard and Riollot, 2003). La caractérisation du pouvoir bactéricide des neutrophiles pourrait donc constituer un indicateur de plus ou moins grande susceptibilité aux mammites.

### **Les rôles des neutrophiles bovins lors d'une infection mammaire chez la vache**

La mammite est toujours l'un des problèmes les plus importants pour l'élevage laitier ; ainsi, étudier le comportement des neutrophiles du CMH-IIpos au cours de la mammite apporterait de nouvelles perspectives sur la physiopathologie de cette maladie coûteuse. Capitalisant sur les résultats de BoNeutro, notre équipe a obtenu une bourse APIS-GENE pour le projet

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<sup>4</sup> <https://www.filiere-laitiere.fr/fr/chiffres-cles/filiere-laitiere-francaise-en-chiffres> (CNIEL)

Masticells (coordonné par le Dr P. Germon, je suis la co-responsable du WP2 sur le rôle des neutrophiles). Nous avons également obtenu une bourse doctorale CIFRE pour Marion Rambault (qui a effectué son stage de Master2 sur le projet BoNeuro sous ma direction). Dans le cadre de son projet de doctorat, Marion Rambault a réussi à mettre au point des protocoles pour travailler avec du lait inflammatoire et à optimiser plusieurs panels de cytométrie en flux pour le bovin. Elle a analysé le recrutement des neutrophiles chez la vache laitière lors de mammites cliniques et subcliniques. Ses résultats principaux sont :

- Les neutrophiles MHC-IIpos circulent de manière similaire dans le sang des vaches Holstein et Normande à l'homéostasie (les deux principales races laitières en France).
- Les neutrophiles MHC-IIpos non triés affichent une capacité phagocytaire et une production de ROS plus élevées que les neutrophiles MHC-IIneg
- Les neutrophiles CMH-IIpos avec des activités microbicides élevées sont recrutés dans la glande mammaire
- Les ratios de neutrophiles MHC-IIpos et MHC-IIneg circulant dans le sang et recrutés dans le lait ne varient pas au cours d'un épisode de mammite clinique (cinétique étudiée : j0, j4 et j21 post-diagnostic)
- Les neutrophiles régulateurs CMH-IIpos sont présents dans le lait pendant une mammite subclinique et sont positivement corrélés aux lymphocytes T.

Un manuscrit illustrant et discutant de ces résultats est actuellement disponible sur BioRxiv, et en cours de révision dans le Journal of Dairy Science (Rambault et al., 2022).



**C. Tableau résumant mes publications (pour la liste complète, voir la partie Annexe de ce manuscrit).**

Position	Nombre d'articles	Journal et année de publication
1 <sup>ère</sup>	7	INRAE Prod. Animales (2022, in press), Frontiers in Vet. Science (2021) Frontiers Immunol (2019), ISME J (2017), Eur. J Immunol (2016 et 2011), PloSONE (2012)
Dernière	2 + 1 soumis	J. Dairy Science (soumis, disponible sur BioRxiv), Frontiers Immunol (2021), Methods Mol Biol (2021)
2 <sup>ème</sup>	2	FASEB J (2016), PloSONE (2010)
Avant-dernière	1	Frontiers Physiol (2018)
Autre position	7	FASEB J (2021), Journal of Immunology (2021), Vaccines (2021, 2020), Frontiers Immunol (2019), Nat Commun (2014), Vet Res (2011)
<a href="https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date">https://pubmed.ncbi.nlm.nih.gov/?term=Remot+A&amp;sort=date</a> ORCID ID : <a href="https://orcid.org/0000-0001-9896-3216">0000-0001-9896-3216</a>		

**D. Tableau de mes projets financés depuis mon recrutement en tant que chargée de recherche (en bleu les projets terminés, en jaune les projets en cours)**

Homme Mois	Financier	Acronyme et titre du projet	Nom du coordinateur	Début - fin	Mon rôle
6	Appel à projet Jeune Chercheur 2015, INRAE, Dept Santé animale	BIIMbo: Bovine Innate Immunity against <i>Mycobacterium bovis</i>	Aude Remot	2016 - 2017	<b>Coordinatrice</b>
2	EGER 3.0 2017-2021 APIS-GENE	HealthyCalf: Calf health and innate immune biomarkers of susceptibility to infections	Fabrice Laurent (INRAE)	2017-2021	Responsable du WP4
6	Appel à projet Jeune Chercheur 2015, INRAE, Dept Microbiologie	LIMBO: Lung innate immunity against <i>Mycobacterium Bovis</i>	Aude Remot	2017 - 2018	<b>Coordinatrice</b>
6	Institut Carnot	BoNeuro: Bovine Neutrophil, a biomarker of robustness against mastitis	Aude Remot	2018-2019	<b>Coordinatrice</b>
3	Hubert Curien Program (PHC Ulysses)	ONE-TB: the study of bovine tuberculosis through a One Health approach	Aude Remot & Stephen Gordon (UCD)	2018	<b>Co- Coordinatrice</b>
3	ANR AAP2017 Défi 5, Axe 2 - PRC	EpiLungCell: Establishment of <u>Epithelial Lung cell</u> lines for pathogens investigation	Bertrand Pain (INSERM)	2018 - 2021	Responsable de 2 tâches
0.5	INRAE département de Santé Animale	MetaBov: Immunometabolic signatures of <u>bovine</u> innate cells	Delphine Descamps (INRAE)	2020 - 2022	Responsable des travaux en lien avec les neutrophiles
0.5	FEDER, APR-IA 2019 Région Centre Val de Loire	ANIMALT: Reducing the number of animals in research, development of alternative <i>ex-vivo</i> models.	Sonia LACROIX-LAMANDE (INRAE)	2019 - 2022	Responsable des travaux sur le poumon
1.5 (moins après 2021)	FEDER, Program Centre-Val de Loire 2014-2020	IMAG'ISP: Imaging in infectiology	Nathalie Winter (INRAE)	2019 - 2022	Participation à l'imagerie du poumon bovin
2	Hubert Curien Program (PHC Ulysses)	BoNeuViD: Immunomodulation of bovine neutrophils by Vitamin D for control of <i>Mycobacterium bovis</i> infection	Aude Remot & Kieran Meade (UCD)	2021 - 2022	<b>Co- Coordinatrice</b>
2	EGER 4.0 2019-2022 APIS-GENE	MASTICELL: Phenotypic and functional characterization of milk somatic cells involved in mastitis responses	Pierre GERMON (INRAE)	2019 - 2023	Co-responsable du WP2, co-encadrante de thèse CIFRE
1 (moins après 2021)	EU Horizon 2020. INFRAIA-01-2016-2017 call	VetBioNet: Veterinary Biocontained facility Network for excellence in animal infectious disease research and experimentation	Sascha TRAPP (INRAE)	2017 - 2023	Responsable des travaux sur le poumon
0.75	PSPC Pays de Loire, BPI France	Néolac : Nouveaux produits pour la réduction des antibiotiques, la durabilité de la filière LAitière et sa Compétitivité	Gaëtan Véteá Plichart (Biodevas Laboratory)	2020 - 2023	Responsable des travaux en lien avec les neutrophiles
1	Fédération de recherche en Infectiologie (FéRI)	MyCOVID: Comparison of innate lung responses after <i>ex vivo</i> infection by mycobacteria from the <i>Mycobacterium tuberculosis</i> complex and the SARS-CoV-2	Aude Remot & Sébastien Eymieux (INSERM, CHU)	2022 - 2023	<b>Co- Coordinatrice</b>
6 (plus après 2023)	ANR AAP2021, CE20, JCJC	Neuro-bTB: Deciphering the role of bovine neutrophil subsets in bovine tuberculosis	Aude Remot	2022 - 2025	<b>Coordinatrice</b>

## E. Expériences d'encadrement

Inspirer la génération future et créer un climat de recherche dynamique est très important pour moi. L'encadrement d'étudiants et de jeunes collaborateurs est une mission qui me tient à cœur. Je forme des étudiants chaque année et j'essaie de garder contact après leurs passages au laboratoire. Je les aide à poursuivre leur carrière, avec des conseils et des recommandations.

Pour la première fois, je co-encadre une doctorante, Marion Rambault, avec l'aide et la supervision de Nathalie Winter. Je guide Marion tout au long de son doctorat, d'un point de vue scientifique, mais je l'aide aussi à monter en compétences, à se construire un réseau et une carrière dans la recherche.

Le tableau ci-dessous présente les étudiants que j'ai encadrés, et leurs devenirs après leur stage.

Niveau	Nom	Période	Projet	Devenir après le stage
<b>Doctorante</b> (bourse CIFRE)	Marion Rambault	Nov 2019- Fev 2023	Role of neutrophils in mastitis in dairy cows	Doctorante (soutenance le 15/12/2022). Marion veut poursuivre en recherche et cherche un post-doctorat.
<b>Master 2</b> Université de Tours		Jan – Juin 2019	Phenotypic and functional characterization or bovine neutrophils	
<b>Master 2</b> Université de Tours	Théodore Vinais	Jan – Juin 2018	Interactions between bovine neutrophils and <i>Mycobacterium bovis</i>	Theodore est chargé de projet à l'hôpital Esquirol à Limoges
<b>Master 2</b> , Paris V, VII, XI and XII	Azadeh Djavid	Jan-Juin 2010	PAR and TLR expressions in lambs (I co-supervised the practical work only)	Azadeh est pédiatre à Paris
<b>Master 1</b> Université de Tours	Marion Horta	Avr-Mai 2021	Infection of human PCLS with Mb and Mtb	Marion revient l'année prochaine pour un stage de Master 2 avec moi
<b>Master 1</b> Université de Tours	Alexia Tessier	Juin-Juil 2021	Role of neutrophils in mastitis in dairy cow (Marion Rambault a co-encadré Alexia)	Alexia a obtenu son master en juin 2022 et recherche du travail en R&D
<b>Master 1</b> Université de Tours	Leslie Hertereau	Avr-Mai 2021	Transcriptomic signatures of calf alveolar macrophages infected with BCG	Leslie a obtenu son master en juin 2022 et a obtenu un CDD de 2 ans en tant qu'ingénieur d'étude au CNRS.



<b>Master 1</b> Université de Tours	Florian Tomal	Avr-Mai 2017	Interactions between bovine alveolar macrophages and <i>Mycobacterium bovis</i>	Florian est actuellement en thèse dans l'unité (bourse CIFRE, équipe de recherche AIM)
<b>Master 1</b> Université Paris- Saclay	Amélie Riou	Avr-Mai 2015	Isolation and screening of bacteria from broncho-alveolar lavages from allergic children	Amélie a obtenu son master en 2016, actuellement consultante senior - Transformation stratégique et industrielle
<b>Licence professionnelle</b> (3 <sup>ème</sup> année), Université de Tours	Anthony Coupé	Fev-Mai 2021	Interactions between lung epithelial cells and <i>Mycobacterium bovis</i> .	Anthony a obtenu sa licence et est maintenant technicien à l'hôpital de Tours, dans le service d'hématologie.
<b>Licence</b> (2 <sup>ème</sup> année) Orsay University	Alix Penel	Avr-Mai 2014	Isolation and screening of bacteria from SPF neonatal mice lungs	Alix a poursuivi en Master, a travaillé ensuite quelques années en tant qu'assistante de recherche pour les hôpitaux de Paris, et travaille maintenant pour la fondation Adolphe de Rothschild (Chargée de projets cliniques)
<b>DUT</b> Université d'Orsay	Nicolas Garcelon	Avr-Mai 2015	Isolation and screening of bacteria from SPF neonatal mice lungs	Nicolas a fait une école de commerce après sa licence scientifique, il est maintenant consultant en management
<b>Assistant de recherche (CDD)</b>	Elliot Mathieu	Sep-Nov 2015	Crosstalk between lung microbiota and lung cells in mice	Elliot a fait sa thèse dans l'équipe à la suite de son CDD (bourse CIFRE), il est depuis manager de production en métagénomique fonctionnelle pour MetaGenoPolis
Etudiante vétérinaire (3 <sup>ème</sup> année) University college Dublin	Roxane Pépin- Donat	Juin 2019	Analysis of lung explants responses to various TLR ligands	Roxane a fini ses études, et travaille maintenant en tant que vétérinaire dans les laboratoires de Touraine

## F. Expériences d'enseignements

La formation et la transmission des connaissances font partie de mes activités. Bien que mon ambition ait toujours été de devenir chercheuse, j'aime enseigner l'immunologie à des élèves de différents niveaux. Je suis intervenue dans différents enseignements en immunologie, mes élèves étaient :

- En 1ère et 2ème année d'agronomie à AgroParisTech (pendant ma thèse). J'ai donné un cours magistral sur l'étude des réponses immunitaires contre le VRS, deux années de suite.
- En Master 1 à l'université de Cergy Pontoise (vacations pendant mon post-doctorat). Pendant 2 ans, j'ai eu la coresponsabilité de l'unité d'enseignement d'immunologie moléculaire pour les étudiants de Master 1 à l'université de Cergy-Pontoise. J'ai conçu le programme du module avec une collègue, assuré la moitié des cours magistraux et des travaux dirigés (5 CM, 3 TD), conçu l'examen et corrigé les copies.
- En 3ème année de médecine à l'hôpital Saint Antoine à Paris (pendant mon post-doctorat). J'ai donné un cours magistral, et encadré un groupe d'étudiant pour la conception d'un projet de recherche (projet tutoré, 2 x ½ journée). J'ai fait partie du jury pour les soutenances orales.
- En Master 2 à l'université de Tours (depuis mon recrutement CR). Je donne un cours magistral d'1h30 dans le module d'immunité des muqueuses. Je dispense ce cours en anglais, pour les étudiants du Master national I<sup>2</sup>VB et du Master international IDOH. Depuis l'année dernière, je fais aussi une demi-journée de travaux pratiques sur les PCLS bovines pour les étudiants du Master IDOH.
- En juin 2022, j'ai initié des enfants de 3 à 12 ans aux Sciences et à l'Immunologie, à l'école maternelle et élémentaire de Semblancay, près de Tours. Cette intervention s'inscrivait dans le cadre d'un événement organisé par la Société Française d'Immunologie, pour favoriser la curiosité scientifique auprès des plus jeunes (1h par classe, 8 classes visitées).

En plus de l'enseignement ponctuel, j'apporte également mon expertise en tant que membre de jury chaque année pour l'évaluation et la soutenance des mémoires des étudiants de Master 1 et Master 2.

Je suis également sollicitée pour des comités de thèse (Comité de Suivi Individuel), jusqu'à présent, j'ai suivi :

- Damien Garrido, qui a travaillé sur les réponses immunitaires des poulets contre le virus de la grippe et les *E. coli* aviaires. Il a soutenu son doctorat à l'Université de Tours en 2018.
- Rea Bingula, qui a travaillé sur l'axe intestin-poumon dans le cadre du cancer du poumon et de la stimulation avec des prébiotiques. Elle a obtenu son doctorat à

l'université de Clermont Auvergne en 2019.

- Maud Rouault, qui travaille à l'ONIRIS (école vétérinaire de Nantes) sur l'identification des biomarqueurs immunologiques de sensibilité et de sévérité des veaux aux bronchopneumonies. Elle a commencé son doctorat en décembre 2021, et nous nous sommes réunis pour son 1er comité en juin.
- Angélique Corset, qui travaille à Rennes (entreprise INRAE St Gilles & Biodevas) sur les stratégies nutritionnelles pour moduler le statut anti-oxydant et immunitaire des vaches laitières. Elle a commencé sa thèse en août 2021 et son premier comité de thèse s'est tenu à Nouzilly en juin 2022 quand Angélique est venue 3 semaines pour réaliser des expériences dans notre équipe.

J'ai été membre de jury de thèse à deux reprises, j'ai été examinatrice pour le doctorat en sciences de Rea Ringula en 2019 (Influence du cancer du poumon via la stimulation prébiotique du microbiote intestinal), et examinatrice pour la thèse de pharmacie de Myriam Slimi en 2017 (Propriétés et caractéristiques du microbiote, des causes de la dysbiose et l'impact sur les facteurs de risque cardiovasculaires tels que l'obésité et l'hypertension). Ces deux expériences ont été très enrichissantes.



## G. Perspectives

Pour les années à venir, mon objectif principal sera l'analyse du rôle des sous-populations de neutrophiles bovins. J'ai décroché un contrat ANR JCJC sur ce sujet, et je viens de déposer un dossier de thèse pour obtenir une bourse pour 2023 (INRAE/Région Centre Val de Loire ou bourse nationale MRT).

### Comprendre le rôle des sous-populations de neutrophiles dans la physiopathologie de la bTB (ANR JCJC Neutro\_bTB 2022-2025)

Les interactions précoces entre les neutrophiles bovins et Mb, ainsi que les interactions des neutrophiles avec d'autres types de cellules, sont essentielles pour l'élimination des bacilles avant le début de la réponse adaptative, comme observé chez l'homme (Martineau et al., 2007) et pourraient également expliquer pourquoi dans les troupeaux certains animaux fortement exposés ne développent pas de test cutané positif (Cassidy et Martineau, 2014). Aux phases tardives de l'infection, c'est-à-dire lorsque les lésions (granulomes) sont déjà établies au début de l'immunité adaptative, les animaux deviennent généralement positifs au test cutané et doivent être éliminés selon la loi Européenne. Cependant, selon le degré de confinement des bacilles à l'intérieur des granulomes, les animaux peuvent avoir excrété des bacilles dans l'environnement avant leur retrait. Les neutrophiles bovins pourraient également jouer un rôle important dans le développement du granulome, comme montré dans la tuberculose humaine. Mes collègues et moi avons été invités à publier une revue dans le journal *Frontiers in Immunology*, et avons discuté du rôle clé des neutrophiles et des changements dans l'environnement hypoxique dans le contexte de la tuberculose (Remot et al., 2019). Les neutrophiles peuvent également avoir un impact sur l'excrétion de bacilles dans l'environnement pendant la bTB (Cassidy, 2006 ; Palmer et al., 2022).

J'ai émis l'hypothèse que les deux sous-populations de neutrophiles MHC-IIpos et MHC-IIneg que nous avons identifiées jouent des rôles différents au cours de l'infection par Mb chez les bovins. Mon projet ANR JCJC Neutro-bTB, dont l'objectif est de décrypter le rôle des sous-populations de neutrophiles bovins dans la bTB, vient de démarrer en mai 2022.

Le projet est construit autour de deux axes de travail.

Tout d'abord, j'étudierai les interactions précoces entre les sous-populations de neutrophiles bovins et Mb, et j'aborderai les interactions des neutrophiles avec d'autres types de cellules dans l'environnement pulmonaire *ex vivo* en utilisant le modèle PCLS (Remot et al., 2021). Nous émettons l'hypothèse que les neutrophiles MHC-IIpos et MHC-IIneg se comporteront différemment en réponse à l'infection par Mb et/ou interagiront différemment avec d'autres cellules de leur environnement proche dans le poumon (Doz et al., 2013 ; Lombard et al., 2016).

Cette première partie abordera les deux questions clés :

- Comment les neutrophiles bovins réagissent-ils à l'infection Mb et éliminent les bacilles ? Les deux sous-populations sont-elles différentes ?
- Comment l'environnement pulmonaire des bovins active-t-il les neutrophiles ? Les deux sous-populations réagissent-elles différemment ?

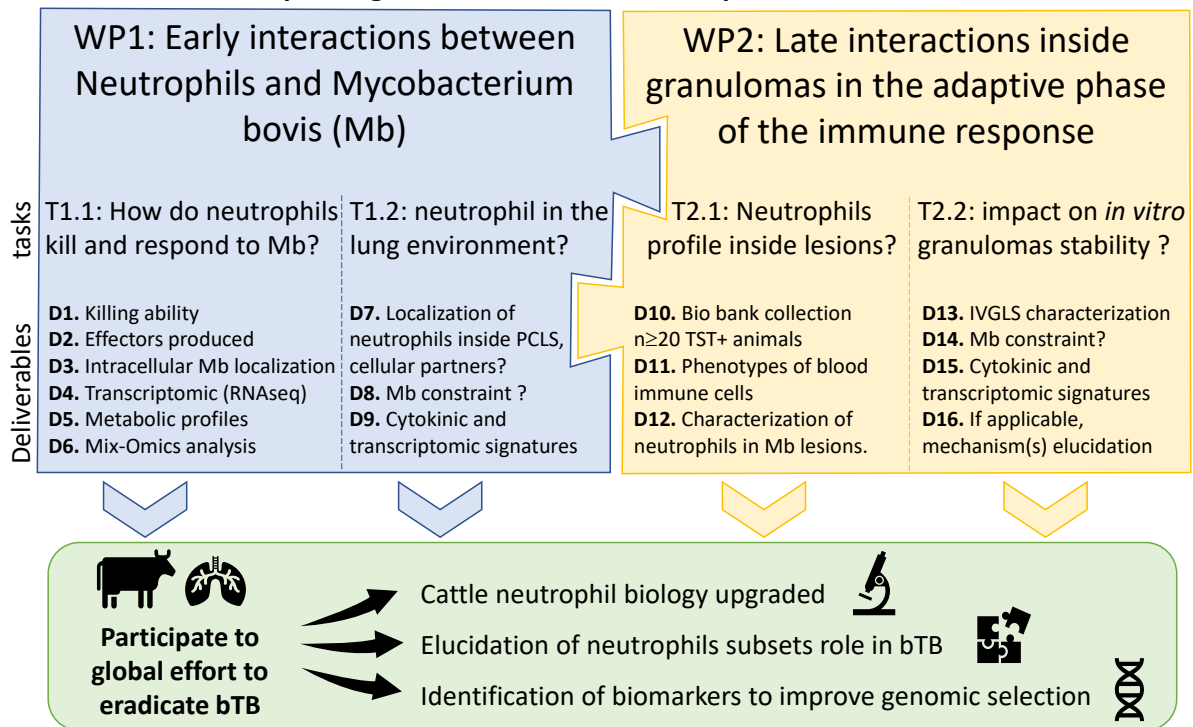
Dans la seconde partie de mon projet, j'étudierai les rôles que jouent les neutrophiles lors des phases tardives de l'infection, lorsque les lymphocytes T CD4+ et les lymphocytes T  $\gamma\delta$  produisant l'IFN $\gamma$  sont recrutés sur le site de l'infection. Notre hypothèse est que les neutrophiles MHC-IIpos modifient le confinement de Mb alors que MHC-IIneg réduit la charge de Mb et favorise l'intégrité du granulome.

Cette seconde partie abordera les questions clés suivantes :

- Quel est le profil des neutrophiles à l'intérieur des lésions bTB chez les bovins ?
- Les neutrophiles MHC-IIpos altèrent-ils la stabilité du granulome ?

J'ai établi les collaborations nécessaires avec le Pr. V. Blanquet (Université de Limoges) pour avoir accès à des échantillons de terrain, d'animaux contaminés par Mb, et avec le Pr. A. Dorhoi (Friedrich Loeffler Institute, Allemagne) qui a établi un modèle de granulome *in vitro* (Agrawal et al., 2018).

## Neutro-bTB: Deciphering the role of bovine neutrophil subsets in bovine tuberculosis



Organisation du projet Neutro-bTB. Les livrables sont résumés en anglais sous chaque tâche.

L'éradication de la bTB est une priorité. La mauvaise compréhension de la physiopathologie de la bTB chez les bovins et le manque de corrélats de protection sont des lacunes importantes dans les connaissances qui doivent être résolues afin de mieux lutter contre la maladie. Le projet Neutro-bTB déchiffrera le rôle des sous-populations de neutrophiles dans la physiopathologie de la bTB chez les bovins. La sélection génomique a eu beaucoup de succès chez les bovins et c'est une voie prometteuse pour améliorer la santé des bovins dans le contexte des maladies infectieuses (par exemple, la variabilité génétique de la résistance aux mammites est bien établie chez les bovins laitiers) (Boichard et al., 2016 ; Rupp et Boichard, 2003). Nous pensons que comprendre le rôle des sous-populations de neutrophiles dans la bTB pourrait conduire à la découverte de nouveaux biomarqueurs susceptibles d'améliorer les programmes de sélection génomique bovine. Chez l'homme, le contrôle génétique de la sensibilité ou de la résistance à la tuberculose est multifactoriel. Bien que les cellules impliquées dans les résultats cliniques de l'infection tuberculeuse restent incomplètement élucidées, les neutrophiles sont des candidats de premier plan (Kroon et al., 2018). Par conséquent, les résultats du projet Neutro-bTB pourraient aider à identifier des biomarqueurs de résistance ou sensibilité à la bTB chez les bovins. Étant donné que ces



informations sont encore indispensables pour les programmes de sélection génétique bovine, le projet Neutro-bTB pourrait grandement contribuer à l'effort mondial d'éradication de la bTB.

Le test cutané de sensibilité à la tuberculine (TST), qui est à la base du contrôle de la bTB chez les bovins, est la signature de l'infection productive et devient positif au début de l'immunité adaptative lorsque les lymphocytes T sensibilisés produisent de l'IFN $\gamma$  en réponse aux antigènes mycobactériens. Si les bovins devaient être sélectionnés pour une meilleure résistance innée à l'infection par Mb - éventuellement grâce à des neutrophiles plus efficaces - les bovins rencontrant Mb élimineraient les bacilles et resteraient négatifs pour le test cutané. Cela entraînerait moins d'abattage d'animaux et moins de pertes pour les éleveurs, ce qui profiterait grandement aux éleveurs et à la santé des animaux ainsi qu'à l'économie. L'amélioration de la résistance à la bTB pourrait également entraîner une diminution de l'excrétion de Mb chez le bétail.

Les neutrophiles sont des cellules immunitaires clés pour l'élimination précoce d'un large éventail d'agents pathogènes envahissants chez les bovins. Nos résultats seront également une grande avancée pour l'immunologie vétérinaire car ils permettront non seulement de mieux comprendre la physiopathologie de la bTB, mais contribueront également à une meilleure compréhension de la biologie des neutrophiles bovins en général.

#### Rôle des neutrophiles dans d'autres pathologies (mammites)

Une meilleure compréhension de la manière dont la réponse immunitaire est orchestrée localement en réponse à une infection respiratoire bénéficiera également à la santé des bovins. Les maladies respiratoires bovines constituent le « complexe respiratoire bovin » : les animaux sont sensibles à une variété de virus et de bactéries respiratoires, en particulier pendant le transport (Earley et al., 2017). Les neutrophiles sont fortement impliqués dans la pathogenèse de cette maladie complexe (Murray et al., 2016). Les nouveaux outils développés par le projet Neutro\_bTB apporteront des avancées importantes dans la compréhension et le traitement futur des maladies respiratoires bovines (et éventuellement déboucheront sur de nouvelles collaborations).

Nos résultats s'appliqueront également à la gestion des mammites. Il est bien établi - en particulier par l'équipe IBIR - qu'un afflux précoce et bien contrôlé de neutrophiles dans la glande mammaire lors de l'invasion par un agent pathogène est le mécanisme prédominant pour la protection (Rainard et Riollet, 2003).

Le projet Masticells se termine en 2023 mais un autre projet pourrait être soumis à APISGENE pour poursuivre nos investigations, avec potentiellement un focus sur les interactions entre les cellules immunitaires et *Streptococcus Uberis*, un des trois principaux agents responsables de mammites.

Je suis actuellement impliquée dans un autre projet de recherche qui vise à objectiver l'effet d'un nouveau produit nutritionnel sur la santé des bovins, avec un intérêt pour la modulation des cellules immunitaires, dont les neutrophiles. Le **projet Neolac** vient de démarrer en août 2021 et est financé par BPI-France, et les régions Pays de la Loire et Bretagne. Ce vaste projet ambitionne de fournir de nouveaux produits nutritionnels pour réduire l'utilisation d'antibiotiques chez les vaches laitières et améliorer la qualité du lait. Le consortium est composé de :

- Biodevas : une PME produisant des produits d'origine végétale, testés comme additifs nutritionnels pour les vaches laitières.
- SODIAAL: la 1ère coopérative laitière de France, qui pilote les essais sur le terrain dans 100 élevages, et contrôle également la qualité du lait (et des produits dérivés du lait) des animaux supplémentés.
- INRAE: 3 équipes de centre différents (Rennes, Clermont-Ferrand, Nouzilly) étudient le mode d'action des produits et encadrent deux protocoles expérimentaux *in vivo* pour objectiver l'effet des solutions nutritionnelles sur la santé animale (statut antioxydant, analyse des réponses immunitaire à la suite d'un challenge inflammatoire avec du LPS, etc.).

Ma contribution est d'étudier la modulation des réponses immunitaires entre les lots d'animaux, en particulier la proportion de sous-populations de neutrophiles, la production de ROS et les capacités phagocytaires.

Il est très important pour moi de continuer à travailler sur les deux maladies d'intérêt pour mon équipe (bTB et mammites), et le rôle des neutrophiles est un très bon moyen pour cultiver la transversalité dans l'équipe.

### Modèle PCLS : une bonne visibilité et des collaborations

Pendant la crise du Covid19, une équipe de recherche du CHU de Tours nous a contactés pour mettre en place des expériences PCLS humaines, afin d'étudier les interactions entre SRASCov2 et les cellules pulmonaires, dans un environnement pulmonaire préservé. Nous avons mis en place un « workflow » (entre nos deux équipes de recherche, et deux services du CHU de Tours), et signé une convention entre le CHU de Tours et INRAE. Notre projet, intitulé Mycovid en clin d'œil à la fois aux Mycobactéries (Mb et Mtb) et au Covid19, a été récompensé par un prix de 10k€ pour le meilleur projet collaboratif, par la Fédération de Recherche en Infectiologie cet été. Mes collaborateurs et moi venons de commencer à analyser les réponses pulmonaires à Mb, Mtb et SRAS-Cov2. Je dirige la partie mycobactéries. J'ai pour objectif de comparer les résultats obtenus sur les PCLS bovines infectées par Mb vs Mtb ; avec ceux des PCLS humaines, pour comprendre les spécificités des réponses en fonction de l'hôte. Mes collaborateurs et moi ambitionnons de publier nos premiers résultats l'année prochaine.

Le modèle PCLS a également été une accroche pour démarrer une collaboration avec le Pr. S. Gordon à l'UCD, co-auteur de notre article (Remot et al., 2021), avec lequel j'interagis beaucoup depuis mon arrivée à Nouzilly. J'ai présenté nos résultats lors de la conférence internationale sur Mb à Galway en juin 2022. Une autre équipe, travaillant à l'université d'Aberystwyth, m'a approchée pour commencer à travailler ensemble, et utiliser notre modèle pour cribler des mutants de BCG et élucider des phénotypes bactériens. Le Dr. A Gibson viendra cet hiver pour réaliser des expériences avec moi, et je leur rendrai visite l'année prochaine pour profiter de leurs installations (Seahorse Cell Metabolism Technology). J'attends avec impatience cette nouvelle collaboration, qui élargira mon réseau hors de France.



## 10. References

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