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1 Comparative impact of porcine reproductive and respiratory virus and swine influenza A 2 virus infections on respiratory lymph nodes B cells and macrophages.

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4 C. Hervet¹, A. Perrin¹, P. Renson², C. Deblanc², M. Muñoz^{3,4,5}, F. Meurens^{1,6,7}, J. Argilaguet^{3,4,5}, G.
5 Simon², O. Bourry², P. Maisonnasse¹, N. Bertho¹

6 ¹ Oniris, INRAE, BIOEPAR, 44300, Nantes, France

7 ² Swine Virology Immunology Unit, Ploufragan-Plouzané-Niort Laboratory, French Agency for Food,
8 Environmental and Occupational Health and Safety (ANSES), 22440, Ploufragan, France.

9 ³ Unitat Mixta d'Investigació IRTA-UAB en Sanitat Animal, Centre de Recerca en Sanitat Animal (CRSA),
10 Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain.

11 ⁴ Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Programa de Sanitat Animal, Centre de Recerca en
12 Sanitat Animal (CRSA), Campus de la Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Spain.

13 ⁵ WOAHC Collaborating Centre for the Research and Control of Emerging and Re-Emerging Swine Diseases in
14 Europe (IRTA-CReSA), 08193 Bellaterra, Spain.

15 ⁶ Department of Veterinary Microbiology and Immunology, Western College of Veterinary Medicine, University
16 of Saskatchewan, Saskatoon, SK, Canada.

17 ⁷ CRIPA, Fonds de Recherche du Québec, Département de pathologie et microbiologie, Faculté de médecine
18 vétérinaire, Université de Montréal, Saint-Hyacinthe, QC, Canada.

19 20 21 **Abstract**

22 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) strongly impacts the pig rearing
23 sector due to its persistence in infected animals. Interestingly, although the PRRSV family exhibits
24 considerable genome variability, with the PRRSV-1 and PRRSV-2 subtypes having been finally
25 classified in two different species (*Betaarterivirus* suid 1 and 2). Both viruses, as well as their derived-
26 attenuated vaccine strains, persist for months, due in part to their ability to delay the appearance of
27 neutralizing antibodies. Thanks to extensive efforts over the past years, we have developed the
28 capability to perform in-depth analysis of the previously poorly understood porcine inverted lymph
29 node (LN). In this study, by comparing the early stages of LN B cell maturation upon PRRSV-1
30 infection to those induced upon the acute swine influenza A virus infection, we highlighted PRRSV-
31 specific mechanisms, including the expression of PD-L1 in efferent macrophages, the induction of
32 extrafollicular plasmacytes, and the influx of inflammatory monocytes/macrophages. Studies on
33 PRRSV-2 infections report observations compatible with our results, that thus might be generalized
34 to all PRRSV-strains. Moreover, these mechanisms can be compared with those used by the human
35 immunodeficiency virus (HIV) and the murine chronic lymphocytic choriomeningitis virus (LCMV) to
36 hijack the immune response. These similarities can be harnessed to develop new strategies to
37 improve the development of more efficient anti-PRRSV vaccines.

38 39 **Introduction**

40 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is an enveloped positive-stranded
41 RNA virus from the *Arteriviridae* family with a tropism for macrophages (MΦ). PRRSV can be divided
42 into two species, *Betaarterivirus* suid 1 (PRRSV-1) and *Betaarterivirus* suid 2 (PRRSV-2), which share
43 60% nucleotide identity (1). The virus causes reproductive disorders in sows and respiratory diseases
44 in piglets. Moreover, the virus' persistence facilitates respiratory super-infections, leading to an
45 important economic impact (2, 3) and an important issue from a One Health perspective (4).
46 Moreover, some emerging American, Chinese, and European (such as Lena) strains are highly
47 pathogenic and threaten the West European pig industry (5–7). Highly pathogenic strains are
48 characterized by high viral loads, severe general clinical signs and high mortality, in sows, weaners
49 and growers.

50 PRRSV persistence mechanisms are still debated. Among the different PRRSV strains from PRRSV-1
51 and -2 species, some induce strong Th1 response (PRRSV 1.2 Lena), some induce very little T cell
52 response (PRRSV 1.1), and others induce a strong Treg response (PRRSV-2) (8–10). This heterogeneity
53 in T helper induction among PRRSV strains suggests that T cell responses are not critical for PRRSV
54 persistence in newly infected adult animals. PRRSV-mediated thymus T cells differentiation
55 perturbations have been demonstrated in newborn piglets (11, 12), which can explain the much
56 stronger impact of PRRSV infection on young animals. However, this can hardly be generalized to
57 adult pigs, which are much less dependent on thymic emigrants to mount an efficient immune
58 response. All PRRSV strains delay the appearance of neutralizing antibodies (NAb) up to one-month
59 post-infection (pi)(13), in both newborn and adult pigs, whereas the non-NAb production occurs
60 within the expected time frame of one-week pi (13). By comparison, swine influenza A virus (swIAV),
61 another major porcine respiratory pathogen, evokes NAb by one-week pi (14). Interestingly, it has
62 been previously observed that unlike swIAV, PRRSV infection using PRRSV-2 and germfree piglets (15)
63 triggers proliferation of germline-encoded B cells of all isotypes, bearing hydrophobic heavy chain
64 CDR3, with little evidence of immunoglobulin diversification (16, 17). It has been observed that the
65 virus, while no longer present in the lung, can be detected in lymph nodes (LN) for months pi. These
66 data led us to interrogate the impact of PRRSV on the B cell maturation process in the very place
67 where it occurs: the LN.

68 To fulfill this task, we first had to better understand the peculiar structure of the porcine inverted LN
69 (18). In a previous work (Bordet et al., 2019), we set up flow cytometry gating and sorting strategies
70 that allowed us to define three LN MΦ populations, to which we tentatively assigned mouse
71 counterparts. The perifollicular MΦ (pfMΦ) are positioned in contact with B cell follicles and have a
72 CD163^{neg}/CD169^{pos} phenotype similar to the murine subcapsular sinus MΦ. A CD163^{pos}/CD169^{neg} MΦ
73 population which we named cord MΦ due to their phenotypic similarity with murine cord MΦ. They
74 are positioned in what we initially identified as the LN cord. It later appeared that these
75 CD163^{pos}/CD169^{neg} cells were localized in the trabecula (the collagen structure supporting the
76 lymphatic sinuses), the lymphatic sinus itself and the T cell area, probably representing different MΦ
77 types. For better accuracy, herein we will name them CD163^{pos}/CD169^{neg} MΦ. The last MΦ
78 population is CD163^{pos}/CD169^{pos} is positioned in the LN parenchyma, at the periphery of the LN
79 corresponding, in inverted LN, to the LN exit. We therefor named it efferent MΦ (effMΦ). We also
80 set protocols to follow B cells positioning and maturation process (Bordet et al., 2019).

81 In the present work, we used this toolbox to compare the early divergences between anti-PRRSV and
82 anti-swIAV innate and adaptive immune responses in the tracheobronchial LN. We opted for an in-
83 depth LN analysis, which required the culling of the animals, but prevented the possibility to conduct
84 a time course study. We have therefore chosen to analyze the LN at 8 dpi, when the most contrasted
85 anti-swIAV and anti-PRRSV immune responses might be expected. At this time, a well-established
86 anti-swIAV immune response can be anticipated, with the appearance of the first NAb. In contrast,
87 the PRRSV infection might have had the time to set up its mechanisms of suppression of the immune
88 response.

89

90 **Material and Methods**

91 *Virus Production and Titration*

92 The highly pathogenic Lena PRRSV-1.3 strain was kindly provided by Dr. Hans Nauwynck, (University
93 of Ghent, Belgium). Lena has been isolated in Belarus in 2007 from a herd with mortality,
94 reproductive failures and respiratory disorders (7). The Lena viral stock for *in vivo* experiment was
95 produced using fresh primary alveolar macrophages (AM) collected from adult animals from the
96 controlled-PRRSV free INRAE herd of the Unité Expérimentale de Physiologie Animale de l'Orfrasière
97 (UEPAO, INRAE, Nouzilly, France). Supernatants from infected cells were clarified by centrifugation at

98 3,300g, filtered on 0.8µm. Virus titration was performed on fresh primary AM using the
99 Ramakrishnan TCID50 method (21).

100 The european human-like reassortant swine H1N2 (swIAV H1N2) strain A/swine/Ille-et-
101 Vilaine/0415/2011 was selected among the collection of the French National Reference Laboratory
102 for Swine Influenza (ANSES, Ploufragan, France). It was isolated from a nasal swab taken from a pig
103 with acute respiratory disease in a herd located in Brittany, France. It was propagated on Madin-
104 Darby Canine Kidney (MDCK) (ATCC reference CCL-34) cells for 24 hours (h) in DMEM medium
105 supplemented with 10% Fetal calf serum, 1% of Streptomycin/Penicillin/Amphotericin solution
106 (Eurobio scientific, Les Ulis, France), and 2 µg/mL of trypsin TPCK (Worthington Biochemical Corp.,
107 Lakewood, NJ, USA). The supernatant was then collected, clarified by centrifugation (600× g, 20 min)
108 and stored at -80°C.

109 Both virus stocks were concentrated and purified on Amicon Ultra-15 centrifugal Filters (Sigma-
110 Aldrich – reference number UFC910024 – pore size 100 kDa Nominal Molecular Weight cut-off) after
111 a 20 min centrifugation at 4000× g and 4°C. Titer determinations of swIAV H1N2 were carried out on
112 MDCK using Reed & Muench method (22). The viral titers of purified swIAV H1N2 reached $9.8 \cdot 10^6$
113 TCID50/mL, while PRRSV-1 stock titer was $2 \cdot 10^9$ TCID50/mL.

114

115 *Animals, in vivo infections and tissue collection.*

116 Infection experiments were conducted at CRESA (Barcelona, Spain). The treatment, housing, and
117 husbandry conditions conformed to the European Union Guidelines (Directive 2010/63/ EU on the
118 protection of animals used for scientific purposes). Animal care and procedures were in accordance
119 with the guidelines of the Good Laboratory Practices under the supervision of the Ethical and Animal
120 Welfare Committee of the Universitat Autònoma de Barcelona (number 1189) and under the
121 supervision of the Ethical and Animal Welfare Committee of the Government of Catalonia (number
122 5796). Twenty pigs (7–8 weeks old, LandracexPietrain) were housed in separate isolation rooms and
123 were randomly assigned to three experimental groups of six (Mock and swIAV-H1N2) or eight
124 (PRRSV-Lena) pigs. The animals were seronegative to IAV (ID Screen Influenza A Antibody
125 Competition ELISA, ID-Vet, Grabels, France) and PRRSV (IDEXX PRRS X3 5/S TRIP), and free of swIAV
126 and PRRSV current infection at the start of the experiment, as tested by PCR using qPCR protocols
127 described in (23) for swIAV and RT-qPCR Kit from Applied biosystems (A35751 VETMAX PRRSV
128 DETECTION KIT 100 RXN - Thermo Fisher) for PRRSV. On day 0 (after one week of acclimatization
129 period), 11 weeks old animals were intranasally infected by $1 \cdot 10^6$ TCID50 swIAV-H1N2 ($5 \cdot 10^5$ /ml per
130 nostril) or $1 \cdot 10^6$ TCID50 Lena ($5 \cdot 10^5$ /ml per nostril). Pigs were examined daily; clinical score was the
131 aggregation of four criterions scored from 0 to 3 (**Suppl. Table 1**). Three of them were monitored
132 daily (behavior, respiratory signs, rectal temperature) and one twice a week (body weight). Nasal
133 swabs were collected daily from 0 to 8 dpi in 500 µl Roswell Park Memorial Institute (RPMI) medium
134 and immediately frozen at -80°C. Sera were collected at 0 and 8 dpi and store at -20°C.

135

136 *Anti-swIAV and anti-PRRSV IgG, IgM and NAb detection in sera*

137 The immunoglobulins G (IgG) directed against the swIAV were quantified with the ID Screen®
138 Influenza A Antibody Competition Multispecies kit (Innovative Diagnostics, Grabels, France) following
139 manufacturer's instructions. IgG levels were expressed in inhibition percentage.

140 Anti-swIAV immunoglobulins M (IgM) were detected with the kit ID Screen® Influenza A
141 Nucleoprotein Swine Indirect kit (Innovative Diagnostics, Grabels, France) with a modified protocol
142 using in-house controls and a goat anti-pig IgM antibody HRP conjugate (A100-117, Bethyl—Fortis
143 Life Sciences, Montgomery, TX, USA) at a 1:10000 dilution as a conjugated antibody. IgM levels were
144 expressed in sample-to-positive (S/P) ratios.

145 NAb targeting the H1N2 strain were quantified by a virus neutralization assay as previously described
146 (Deblanc et al, 2020). The NAb titer was determined as the reciprocal of the highest dilution of serum
147 that prevents virus infection of the cell monolayer, determined by the absence of cytopathic effect. A
148 serum was considered positive when virus neutralization titer ≥ 20 .

149 Levels of immunoglobulins G (IgG) directed against PRRSV (N protein) were assessed using the
150 Pigtype PRRS Ab ELISA kit (Indical Bioscience GmbH, Leipzig, Germany) following manufacturer's
151 instructions and expressed as sample-to-positive (S/P) ratios. Anti-PRRSV (N protein) IgM levels were
152 measured using the same PRRSV ELISA kit, replacing the kit's conjugated antibody by a goat anti-pig
153 IgM HRP-conjugated antibody (A100-117, Bethyl Laboratories—Fortis Life Sciences, Montgomery, TX,
154 USA) at 1:25000 dilution and replacing the commercial controls by in-house calibrated negative and
155 positive serum controls to calculate sample-to-positive (S/P) ratios. PRRSV-specific NAb targeting the
156 Lena strain were quantified in serum using MARC145 cells. Heat-inactivated sera were two-fold
157 serially diluted at 56 °C for 30 min, and then 50 μ l of each dilution was incubated in duplicate in 96-
158 well microtiter plates with the MLV1 DV strain at 101 ± 0.5 TCID₅₀/50 μ l for 1 h at 37°C with rocking
159 agitation. A suspension of MARC-145 cells (0.5 $\times 10^5$ per well) was then added. After incubation for
160 five days at 37 °C, the titers were determined as the reciprocal of the highest dilution of serum that
161 prevented virus infection of the cell monolayer, as determined by the absence of cytopathic effects
162 in half of the duplicate wells. A serum was considered positive when virus neutralization titre ≥ 10 .

163

164 *Serum cytokines dosage*

165 Serum cytokines concentrations were determined according to manufacturer instructions for
166 CXCL13, IL-5, BAFF, IL-2, IL-4, IL-6, IL-13 IL-17 and IL-10 and IFN- γ . CXCL13, IL-5 and CD257 were
167 quantified by sandwich ELISA with kits from LSBio (LS-F41718 Porcine BLC/CXCL13 and LS-F45145
168 Porcine IL-5) or from Biosource (MBS2512446 Porcine BAFF/CD257). Other cytokines were measured
169 by 2-plex (Porcine IL-10, IFN- γ) or 5-plex (Porcine IL-4, IL-13, IL-17, IL-6, IL-2) Luminex assays. All kits
170 were purchased to Abyntek Biopharma S.L. (Bizkaia, Spain).

171

172 *Lymph node collection and cells isolation*

173 At 8 dpi, animals were culled and lungs were collected. The tracheobronchial LN in the precisely
174 delimited space between the right main bronchia and the accessory right bronchia were collected for
175 dissociation. LN were minced and incubated with RPMI 1640 supplemented with 100 IU/ml penicillin,
176 100 mg/ml streptomycin, 250 ng/ml Fungizone® (Antibiotic-Antimycotic 100X ThermoFisher
177 Scientific, Illkirch, France), 2mM L-glutamine and 10% heat inactivated fetal calf serum (FCS,
178 Invitrogen, Paisley, UK). Digestion was performed for 30 min at 37°C with 2 mg/ml collagenase D
179 (Roche, Meylan, France), 1 mg/ml dispase (Invitrogen) and 0.1 mg/ml DNase I (Roche). Filtration on
180 40 μ m cell strainers was performed and red blood cells were lysed using erythrolysis buffer (10mM
181 NaHCO₃, 155mM NH₄Cl, and 10mM EDTA). Cells were washed in PBS/EDTA, and re-suspended in FCS
182 + 10% dimethyl sulfoxide (DMSO) for liquid nitrogen storage before cell sorting.

183 The tracheobronchial LN situated at the bifurcation of the two main bronchia were collected for
184 Tissue Teck (Sakura, Paris, France) embedding and were frozen on dry ice before -80°C storage.

185

186 *Flow Cytometry Analysis and Cell Sorting*

187 Cells were stained in a blocking solution, composed of PBS-EDTA (2mM) supplemented with 5%
188 horse serum and 5% swine serum. Staining was made in 4 steps, including PBS/EDTA with 2% FCS
189 washing between each step: uncoupled primary anti-CD169, anti-IgM and anti-CD172a antibodies
190 (**Table 1**) were added to the blocking solution for 30min on ice and then washed. Fluorescent,
191 secondary, mouse isotype specific antibodies were then added, respectively anti-IgG2a-PE-Cy7, anti-
192 IgM-Dye-Light-649 and anti-IgG2b-APC-Cy7 (**Table 1**) for 20 min on ice and then washed. Then, to

193 saturate the potential unbound IgG1-directed secondary antibodies sites, cells were incubated for 30
194 min in the blocking solution supplemented with isotype-control IgG1 (10 µg/ml). Third, the
195 fluorochrome-coupled primary IgG1: anti-CD21 coupled to FITC, anti-CD163 coupled to PE (**Table 1**)
196 were added for 30 min on ice and then washed before re-suspension in a DAPI-containing buffer for
197 sorting. Sorting was performed using a BD FACSAria II cell sorter (Becton Dickinson, San Jose, USA)
198 equipped with 407, 488, and 633nm lasers. Temperature was kept at 4°C during the whole sorting.
199 FlowJo software (version X.1.0, Tree Star, Ashland, OR, USA) was used for analysis. Sorted cells were
200 centrifuged and lysed in RLT buffer for storage at -80°C before further processing.

201

202 *RT-qPCR*

203 Total RNA in RLT Buffer were extracted using RNeasy MicroKit (Qiagen) following the manufacturer's
204 instructions. Total RNA quantity and quality were assessed using Nanophotometer (Implen, Munich,
205 Germany). cDNA were generated with a reverse transcriptase in the iScript Reverse Transcription
206 Supermix for RT-qPCR (Bio-Rad, Hercules, CA, USA). The generated cDNAs were then diluted (2×) and
207 combined with the primer set and SYBR Green Supermix (Bio-Rad) following the manufacturer's
208 recommendations. PRRSV-Lena and swIAV genomes were detected by multiplex qPCR (Eurogentec,
209 Liège, Belgium) with probes and primers specified in **Table 2**.

210 *Immunohistochemical staining*

211 Sections of 14 µm were obtained from Tissue Teck embedded LN using a cryostat (Leica CM3050S,
212 Nanterre, France) and deposited on Superfrost® glass slides (ThermoFisher scientific). Cryosections
213 were fixed in methanol/acetone (1:1) at -20°C for 20 min. Fixed slides were saturated using PBS
214 supplemented with 5% horse serum and 5% swine serum for 30 min at room temperature (RT).
215 Primary and secondary antibodies (**Table 1**) were added at 4°C overnight or for 30 min, respectively.
216 Acquisition was performed using the same settings for all slides to ensure comparable fluorescence
217 parameters. Microscopy cell counting was performed using Zen software (Zeiss). For intra- and
218 interfollicular centroblasts (CB) and centrocytes (CC) numbering, a virtual line was drawn across the
219 follicle, extending 20% out of the follicle's diameter on each side. The follicular area limit was
220 considered reached when two CD21 pixels showed a relative fluorescence above 500. Cells were
221 identified along this line as signals having at least 3 pixels above 250 (for Alexa-555) or 550 (for Alexa-
222 647) of relative fluorescence, representing respectively Bcl6 and Pax5 expressions. LN from 4
223 different animals from the 3 infectious conditions (Mock, PRRSV, swIAV) were analyzed. Two or three
224 follicles per LN were blindly counted.

225 For MΦ numbering, T cell areas were defined as areas between two Pax5-positive follicles and
226 trabeculae were defined as areas with rare DAPI staining along Pax5-positive follicles. LN from 3
227 different animals from the 3 infectious conditions (Mock, PRRSV, swIAV) were analyzed. Three
228 different T cell areas per LN were blindly counted. A fluorescent signal in one of the 2 channels
229 defining MΦ (Alexa-555 and Alexa-647, defining respectively CD163 and CD169 expressions) with a
230 relative fluorescence above 300 and composed of at least 3 pixels, was counted as one cell. For both
231 MΦ and B cells staining, in some areas, it was impossible to distinguish individual cells within dense
232 cell clusters. In such case, a continuous signal exceeding 10 µm was considered to represent 2 cells, a
233 signal above 20 µm represented 3 cells, and so on.

234

235 *Statistics*

236 Graph Pad Prism 10.2.3 (GraphPad Software) was used for graph design and statistics. Shapiro-Wilk
237 test was used to assess the distribution of the data. Between infectious conditions, an unpaired non-
238 parametric Mann-Whitney test was used. When intra-infectious conditions were compared (0 dpi vs
239 8 dpi or different B cells or MΦ populations), a paired non-parametric Wilcoxon rank test was made.

240

241 **Results**

242 *PRRSV Lena triggered higher viral load and clinical signs than swIAV, associated with a lower lymph*
243 *node cellularity and the absence of neutralizing antibodies.*

244 Seven/eight weeks old animals were intranasally infected with a dose of 1.10^6 TCID₅₀ of swIAV-H1N2
245 or PRRSV-1 Lena. Strong swIAV shedding was observed at 2 dpi before decreasing. Influenza virus
246 was no longer detected in nasal swabs by 8 dpi (**Fig. 1A**). Conversely, PRRSV shedding appeared at 2
247 dpi similarly to swIAV. However, PRRSV levels continued to increase at 4 dpi before gradually
248 decreasing by 8 dpi (**Fig. 1A**). As expected, intranasal swIAV infection triggered few clinical signs, that
249 were totally resolved at 6 dpi, whereas the pathogenic Lena PRRSV 1.3 strain triggered clear clinical
250 signs for the 8 infected animals at 3 dpi, with no improvement at 8 dpi (**Fig. 1B**).

251 We then validated the humoral immune response course upon viral infections by measuring serum
252 anti-swIAV and anti-PRRSV IgM, IgG and NAb titers. The 3 types of antibodies directed against swIAV
253 were readily detected at 8 dpi (**Fig. 2A**). Conversely, whereas anti-PRRSV IgM and IgG serum
254 antibodies were present, no anti-PRRSV NAb were observed (**Fig. 2B**).

255 At 0 and 8 dpi, different cytokines and chemokines known to have roles in the B cell differentiation
256 and maturation processes were titrated. No difference between infectious conditions and time
257 points were observed for IL-2, IL-4, IL-6, IL-10, IL-13, IL-17 nor IFN- γ (data not shown). IL-5
258 concentrations at 8 dpi were significantly higher in PRRSV-1 compared to Mock- and swIAV-infected
259 animals and presented a significant increase between 0 and 8 dpi (**Fig. 2C**). Two other cytokines,
260 CXCL13 and BAFF, presented a similar significant increase between 0 and 8 dpi in the PRRSV-infected
261 group but not in the Mock and swIAV groups (**Fig. 2C**).

262

263 *Upon PRRSV Lena infection, centrocytes were reduced in number and presented an perifollicular*
264 *location.*

265 Interestingly, and despite higher viral loads and clinical scores, LN cellularity at culling (8 dpi)
266 indicated a lower intranodal reaction upon PRRSV compared with swIAV infection (**Supp. Fig. 1A**). To
267 note, one swIAV-infected animal has been excluded from the analysis because of its abnormally fast
268 settlement of the influenza infection, with no more virus shedding as soon as 6 dpi (**Supp. Fig. 1B**),
269 associated with a LN cellular count similar to that of the Mock-infected animals at 8 dpi (**Supp. Fig.**
270 **1C**).

271 After LN cell counting, we stained, analyzed and sorted LN M Φ and B cell at different maturation
272 stages by flow cytometry as previously described (19). Namely, CD169^{pos}/CD21^{pos}/IgM^{neg} **CD169^{pos} CB**,
273 CD169^{neg}/CD21^{pos}/IgM^{neg} **CB**, CD21^{low}/IgM^{pos}/FSC^{low} **CC**, CD21^{high}/IgM^{pos} FSC^{high} plasmablasts (**PB**) and
274 CD21^{neg}/IgM^{pos}/CD172a^{neg}/FSC^{high} plasmacells (**PC**) were sorted (**Supp. Fig. 2**). CB sorting was
275 validated on Mock-infected animals using RT-qPCR by the significant over-expression of
276 topoisomerase IIa (Topo2a), a marker of proliferating cells, and of activation-induced cytidine
277 deaminase (AICDA), an enzyme involved in somatic hypermutations (SHM) (**Fig. 3A**). Although non-
278 significant, the expression of Bcl6, a master transcription factor of CB, was higher in CB compared
279 with PB and PC, as expected. Similarly, Blimp1, a marker of terminally differentiated B cells,
280 presented a slightly higher expression in PC (**Fig. 3A**). CXCR5 is the receptor for CXCL13, a chemokine
281 expressed by follicular Dendritic Cells (fDC) and responsible for B cell recruitment in the B cell follicle
282 (28). CXCR5 presented no differential expression among B cell populations of Mock-infected animal
283 LN (**Fig. 3A**).

284 Upon infection, we observed a higher expression of AICDA and Blimp1 in PC from PRRSV- compared
285 with swIAV-infected animals, a lower expression of Bcl6 in CB of PRRSV- and swIAV- compared with
286 Mock-infected animals, as well as a higher CXCR5 expression in PB from PRRSV- compared with

287 Mock-infected animals (**Fig. 3B**). Topo2a presented no significant difference among the infectious
288 conditions (data not shown).

289 To increase the statistical power for comparing gene expression in the different B cell populations,
290 we pooled the data from each population (CD169^{pos} CB, CB, CC, PB, PC) from the different infectious
291 statuses after having excluded the populations in which markers presented significant differences
292 compared to Mock: Bcl6 expressions in swIAV and PRRSV CB, Blimp1 in swIAV PC and CXCR5 in
293 PRRSV PB (**Fig. 3B**). We then observed a significant over-expression of Topo2A, AICDA and Bcl6 in CB
294 (both CD169^{pos} and CD169^{neg}) as well as an over-expression of Blimp1 in PC compared with all other B
295 cell populations, validating further our cell sorting strategy. Interestingly, we also found an over-
296 expression of CXCR5 in CD169^{pos}CB compared with regular CB (**Fig. 3C**).

297 Having validated our sorting strategy, we then enumerated the different B cell populations. As
298 expected by the higher cellularity in LN from swIAV-infected animals, all the B cell populations
299 (CD169^{pos} CB, CB, CC and PB) were over-represented in swIAV-infected compared with Mock- and/or
300 PRRSV-infected animals, with the striking exception of PC, which appeared as numerous in PRRSV-
301 infected as in swIAV-infected animals LN (**Fig. 4A**). We then performed LN tissue immunofluorescent
302 staining (**Supp. Fig. 3**) in order to analyze the intra- or extra-follicular positioning of the different B
303 cell maturation steps. LN from Mock- and swIAV-infected animals offered a classical morphology.
304 However, PRRSV-infected animals LN systematically displayed a disturbed effMΦ area, associated
305 with a lower conservation of the whole LN parenchymal structure (**Supp. Fig. 3**). Despite this
306 difficulty, we were able to localize CD21^{pos} follicles and to count Bcl6^{pos} CB, and Pax5^{pos} CB and CC
307 inside and outside the follicles. Bcl6^{pos} cells were almost exclusively intrafollicular in all conditions
308 (perifollicular Bcl6^{pos} cells: Mock 1%+/- 2%, PRRSV 9%+/-9%, swIAV 2%+/-3%). Pax5^{pos} cells were also
309 mostly perifollicular. However, PRRSV-infected animals presented significantly more perifollicular
310 Pax5^{pos} B cells than swIAV-infected animals (perifollicular Pax5^{pos} cells: Mock 13%+/-11%, PRRSV
311 23%+/-7%, swIAV 16%+/-7%) (**Fig. 4B**).

312

313 *PRRSV Lena infection triggered the incoming of inflammatory monocytes in T cell area.*

314 LN MΦ were defined as CD163^{pos}/CD169^{neg} MΦ, CD163^{pos}/CD169^{pos} effMΦ and CD163^{neg}/CD169^{pos}
315 pfMΦ. We validated the LN MΦ sorting by measuring the expression of previously tested (19) MΦ
316 differentiation genes : CSF1R and MAFB (**Supp. Fig. 4A**). Six sorted populations did not
317 transcriptionally express MΦ markers' positive controls and were excluded from further analysis:
318 Mock-infected animal 12 CD163^{pos}/CD169^{neg} MΦ and effMΦ, Mock-infected animal 13 pfMΦ; PRRSV-
319 infected animal 11 CD163^{pos}/CD169^{neg} MΦ and swIAV-infected animal 7 CD163^{pos}/CD169^{neg} MΦ and
320 eff MΦ. We then tested genes potentially involved in MΦ functions, CCR2, IL-6, iNOS, PD-L1, CCL2
321 and CXCL13, and observed no significant differences between MΦ populations from mock-infected
322 animals (**Supp. Fig. 4B and 4C**). CCL2, a chemokine involved in the attraction of inflammatory cells
323 including blood monocytes (29), presented a tendency to be more expressed in effMΦ compared
324 with CD163^{pos}/CD169^{neg} MΦ and pfMΦ (p=0.06 and p=0.1 respectively) (**Supp. Fig. 4C**).

325 By combining whole LN cell count with the percentage of each population, as measured by flow
326 cytometry, we were able to calculate an absolute number of cells in each MΦ populations. Contrarily
327 to B cells, the higher cellularity in LN from swIAV-infected animals did not significantly impact the
328 MΦ populations in swIAV- compared with Mock- or PRRSV-infected animals LN (**Fig. 5A**). However,
329 CD163^{pos}/CD169^{neg} MΦ were significantly over-represented in LN from PRRSV-infected animals as
330 compared with Mock- (50 times increase) and swIAV- (10 times increase) infected animals (**Fig. 5A**).

331 To localize the LN CD163^{pos}/CD169^{neg} MΦ appearing upon PRRSV infection, we proceeded to a
332 fluorescent microscopy analysis. B cell follicles were identified through Pax5 staining and the
333 CD163^{pos}/CD169^{neg} MΦ population was distinguished through CD163 and CD169 differential
334 expressions (**Supp. Fig. 5**). We localized and counted the CD163^{pos}/CD169^{neg} MΦ present in the

335 trabeculae and sinuses (CD163^{pos}/CD169^{neg} trabecular MΦ) as well as those located in the T cell area
336 between the B cell follicles (CD163^{pos}/CD169^{neg} T cell zone MΦ). Whereas Mock- and swIAV-infected
337 animals presented virtually no CD163^{pos}/CD169^{neg} MΦ in T cell areas, PRRSV infected animals
338 presented a strong influx of these cells (**Fig. 5B and 5C**). In addition, swIAV-infected animals
339 presented less CD163^{pos}/CD169^{neg} MΦ in the trabeculae and the sinuses than PRRSV-infected animals
340 (**Fig. 5C**). Thus, the CD163^{pos}/CD169^{neg} MΦ that appear upon PRRSV-infection are associated with the
341 T cell area rather than the trabecula, and have likely different origins and functions compared to
342 steady state CD163^{pos}/CD169^{neg} MΦ. Indeed, RT-qPCR on sorted MΦ populations highlighted the
343 over-expression of CCL2 gene in CD163^{pos}/CD169^{neg} MΦ from PRRSV-infected animals compared with
344 Mock- and swIAV-infected animals, in agreement with a pro-inflammatory signature (**Fig. 5D**).
345 Conversely, effMΦ from PRRSV-infected animals strongly down-regulated CCL2 compared with
346 Mock- and swIAV-infected animals. When compared between PRRSV and swIAV conditions,
347 CD163^{pos}/CD169^{neg} MΦ presented a higher expression of CCR2, the CCL2 receptor, upon PRRSV
348 infection. CXCL13 expression was not significantly different between MΦ populations or infectious
349 status, although pfMΦ from PRRSV-infected animals presented high CXCL13 expression in 4 out of 6
350 animals (**Fig. 5D**). Finally, effMΦ from PRRSV-infected animals presented an up-regulation of the
351 immune checkpoint-ligand PD-L1 (**Fig. 5D**).

352

353 Discussion

354 Porcine CD169^{pos} centroblasts

355 First of all, this work allowed us to complete and strengthen our previous results on B cell
356 differentiation steps in the swine inverted LN (19, 20). We confirmed our CB identification strategy as
357 both CD169^{pos} and regular CB presented Topo2A and Bcl6 over-expression, as previously validated
358 (19); moreover, they also presented a clear over-expression of AICDA, an enzyme involved in SHM, a
359 strong marker of CB. In addition, we observed for the first time a differential expression between the
360 two CB sorted clusters: CXCR5 presented a higher expression on CD169^{pos} than on regular CB. CXCR5
361 is the chemokine receptor involved in the migration toward CXCL13, the chemokine which gradient
362 allows the shuttling of B cells transporting antigens, as described in mice (30–32). We previously
363 showed that CD169^{pos} CB expressed membrane CD169 in the absence of detectable CD169 mRNA,
364 hypothesizing that this CD169 display might result from CB/pfMΦ membrane exchanges during
365 antigen transfer from pfMΦ to CB (19). CXCL13 is secreted by follicular stromal cells (33–35), creating
366 a gradient attracting CXCR5-expressing cells towards the follicle center through complex interactions
367 with EIB2/7α,25-dihydroxycholesterol, sphingosine-1-phosphate (S1P)/S1P-receptor and
368 CXCL12/CXCR4 (for review (36, 37). Thus, CXCR5 over-expression on CD169^{pos} CB reinforces our
369 hypothesis of a role of these cells in transporting free antigens from pfMΦ (at the follicle periphery)
370 to FDC (in the follicle light zone) (30–32).

371

372 Serum cytokines and extrafollicular centrocytes

373 CXCL13 and IL-5 serum levels have been investigated during HIV chronic infections in human. It has
374 been reported by two groups that CXCL13 serum concentration was positively correlated with NAb
375 responses (38, 39). Conversely, in two other studies, CXCL13 appeared overexpressed in patients
376 with progressive disease compare with elite controller (HIV-infected patients who naturally
377 controlled their viral load) and patients on antiretroviral therapy (40, 41). In this last study, contrarily
378 to CXCL13, IL-5 correlated with nAb breadth (41). In a PRRSV context, it seems that IL-5, CXCL13 and
379 BAFF, are the markers of an unresolved immune response trying to unsuccessfully control the viral
380 load rather than markers of an efficient neutralizing immune response. Accordingly, it would be of
381 great interest to compare the serum expression of these cytokines and chemokines on a daily basis
382 during the early steps of swIAV and PRRSV infections in pigs.

383 Despite ongoing viral shedding and a higher clinical score, we observed lower total LN cellularity in
384 PRRSV- compared to swIAV-infected animals LN. Notably, this deficit was paralleled by CB, CC and PB,
385 but not PC, decreases. One possible explanation for this discrepancy is an extrafollicular
386 differentiation of PC during PRRSV infection. To investigate this possibility, we checked for
387 extrafollicular B cell differentiation and observed an increase in perifollicular Pax5^{pos} B cells upon
388 PRRSV infection. Whether these cells account for extrafollicular B cell differentiation that would skip
389 the intermediate follicular steps and the ensuing Ab affinity maturation (for review (42)), as
390 postulated by others (15, 16), remains to be explored in more detail.

391 Concerning B lymphocytes maturation steps, the only gene expression differences between PRRSV-
392 and swIAV-infected animals were the over-expression of AICDA and Blimp1 in PC. AICDA expression
393 in PC usually reflects isotype switching, which might again be the consequence of the ongoing PRRSV
394 immune response, as might be the Blimp1 overexpression, compared with the resolving anti-swIAV
395 infection. However, the main role of AICDA is to trigger SHM, and SHM occurring in PC have been
396 associated with extrafollicular B cells maturation (43, 44). Thus, the over-expression of AICDA in PC is
397 in agreement with extrafollicular B cells maturation.

398

399 Inflammatory monocytes/macrophages and PD-L1

400 Upon PRRSV infection, we observed an influx of CD163^{pos}/CD169^{neg} monocytes/MΦ in the LN T cell
401 area. According to murine data, at steady state, this MΦ phenotype might include different LN
402 populations, such as medullary cord MΦ and T cell zone MΦ (for review (45)).

403 In the murine model of acute and chronic lymphocytic choriomeningitis virus (LCMV), it has been
404 shown that chronic strains were endowed with the unique capacity to attract inflammatory myeloid
405 cells which inhibit both T (46) and B cell (47) mediated immune responses. In agreement with this
406 model, we observed an increase of CCL2 expression in CD163^{pos}/CD169^{neg} MΦ in LN of PRRSV-
407 infected pigs, consistent with an inflammatory monocyte signature. Interestingly, an influx of
408 uncharacterized MΦ in the T cell zone of pig LN has already been observed with different PRRSV
409 strains, although the pattern was more pronounced with the highly pathogenic SU1-Bel strain (48).
410 According that the Lena strain used here was also highly pathogenic, a relation between pathogeny
411 and LN MΦ influx in the LN can be proposed. Furthermore, while the CD163^{pos} MΦ population
412 increases and up-regulates CCL2 upon PRRSV infection, effMΦ strongly down-regulate CCL2
413 expression. This might lead to the weakening of the CCL2 gradient present in Mock- and swIAV-
414 infected animals, from the T cell area toward the efferent area. The role of this LN gradient in normal
415 and altered immune responses remains to be explored.

416 PD-1, the PD-L1 receptor, is expressed on B cells and acts as an inhibitor of the B cell activation
417 cascade (49). PD-L1 has been shown to be over-expressed in the thymus (27), broncho-alveolar
418 lavage (50), lung tissue, in *in vivo* infected AM (51) as well as in LN (52) of animals infected with
419 different strains of PRRSV-1. We observed a PD-L1 over-expression in effMΦ, the porcine
420 counterpart of medullary MΦ (19), which are thought to aid in the final maturation of PB into PC in
421 the murine model (53). Indeed, the efferent area is the porcine LN region where mature or maturing
422 Blimp-1^{pos} B cells accumulate in close proximity to effMΦ (20). Anti-porcine PD-L1 nAb have been
423 developed (54) and would be worth testing in PRRSV-infectious settings.

424

425 Conclusion

426 Herein, we were able to investigate in detail the differential impact of swIAV and PRRSV infections on
427 B cell maturation. By this PRRSV/swIAV comparison in growing, conventional animals, we comforted
428 two of the main hypotheses previously proposed for both PRRSV-1 and PRRSV-2 independently of
429 pathogenicity level: the likely induction of extrafollicular B cell activation (16, 17) and the over-
430 expression of PD-L1 in different tissue MΦ (27, 50, 52). We also evidenced the invasion of LN T cell

431 areas of PRRSV-infected animals by inflammatory monocytes/MΦ that may have a strong impact on
432 the B cell maturation process, as described for LCMV in mice (47). Although this last feature might be
433 related to the level of PRRSV pathogenicity. Attenuated vaccines are one of the main resources to
434 control PRRSV in farms. However, they still have similar abilities to inhibit the immune system as
435 wild-type PRRSV, which contributes to their low efficacy and raise concerns about their use (55).
436 PRRSV inhibition of the B cell response induction seems multifactorial and it remains to be explored
437 which PRRSV-genes/factors are involved in these different aspects of the immune blocking, in order
438 to design efficient attenuated vaccines depleted of these properties.

439

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446

447 **Authors contributions**

448 CH prepared the viruses for inoculation. CH, MM and NB proceeded the serum, lung and lymph node
449 tissues. JA and NB performed cell sorting. AP and NB carried out immunostaining of LN slides. CH and
450 AP conducted RT-qPCR. PR, CD, GS and OB handled immunoglobulin titrations and neutralizing
451 antibodies determinations. CH, PR, CD, FM, JA, GS, OB PM and NB corrected and edited the
452 manuscript, providing thorough discussions and critical revisions. NB supervised the work, designed
453 the experiments, analyzed the images, prepared the figures and wrote the manuscript. All authors
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455

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461

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634

635 **Figure legends**

636

637 **Figure 1:**

638 **PRRSV Lena and swIAV H1N2 infected pigs present contrasting viral loads and clinical signs.**

639 Pigs were intranasally exposed to either PBS (Mock - black - n=6), 1.10^6 TCID50 of a PRRSV Lena strain
640 (PRRSV - blue - n=8) or 1.10^6 TCID50 of a swIAV H1N2 strain (swIAV - red - n=6). **A)** RNA viral loads
641 measured by RT-qPCR in nasal swabs (means with SD). **B)** Clinical scores (mean with range) were
642 recorded from day 1 to day 7 post-infection. Mann-Whitney unpaired two-tailed t-test, *p* values:
643 * <0.05 , ** <0.01 , *** 0.001 .

644

645 **Figure 2: Anti-PRRSV and swIAV serum antibodies, neutralization and B cell maturation related**
646 **cytokines.**

647 Pigs were exposed to either PBS (Mock - black - n=6), PRRSV Lena strain (PRRSV - blue - n=8) or swIAV
648 H1N2 strain (swIAV - red - n=6). Serum was sampled at 0 (empty) and 8 (full) days post-infection.
649 Individual IgM, IgG and neutralizing antibodies titers against swIAV (**A)** or PRRSV (**B**). IgM levels are
650 represented as S/P ratio. IgG levels are represented as 1/(inhibition percentage). Neutralization titers
651 corresponded to the reciprocal of the highest dilution of serum able to neutralize the virus. Bars
652 represent means. C) IL-5, CXCL13 and BAFF concentrations in serum. Wilcoxon matched-pairs signed
653 rank test, *p* values: * <0.05 , ** <0.01 , *** 0.001 .

654

655 **Figure 3: B cells maturation stages specific gene expressions.**

656 Lymph node cells were stained for macrophage subpopulations phenotyping, analyzed (see **Sup. Fig.**
657 **2**) and sorted using a flow cytometer cell sorter. The relative expression of genes specific of different
658 stages of B cell maturation were measured by RT-qPCR. **A)** B cell maturation stages sorted from
659 Mock-infected animals LN. **B)** Comparative transcriptomic expression of B cell maturation steps
660 according to infectious status (Mock - black, PRRSV - blue, swIAV - red). **C)** Comparative
661 transcriptomic expression of B cell maturation steps with pooled infectious conditions whenever no
662 gene expressions differences were observed in B). Bars represent means.

663 A) and C) Wilcoxon matched-pairs signed rank test, B) Mann-Whitney unpaired two-tailed t-tests, *p*-
664 value: * <0.05 , ** <0.01 , *** <0.001 . CB CD169^{pos}: CD169-positive centroblasts, CB: CD169-negative
665 centroblasts, CC: centrocytes, PB: plasmablasts, PC: plasmacells, AICDA: activation-induced cytidine
666 deaminase, Topo2a: topoisomerase IIa.

667

668 **Figure 4: LN B cells subpopulations count in the complete LN or at specific locations at day 8 post**
669 **PRRSV-Lena or swIAV-H1N2 infections.**

670 A) Absolute B lymphocyte LN numbers at different maturation steps, as calculated from absolute
671 total cell number count (**Supp. Fig.1A**) and B lymphocyte percentages determined by flow cytometry
672 (**Supp. Fig. 2**). B) LN B cells count in the intra-follicular and perifollicular area obtained by microscopic
673 analysis of whole LN (see **Sup. Fig. 3**) stained for CD21, Bcl6 and Pax5. Bars represent means. A)
674 Wilcoxon matched-pairs signed rank test B) Mann Whitney unpaired two-tailed t-tests, *p*-value:
675 * <0.05 , ** <0.01 .

676

677 **Figure 5:**

678 **Lymph node macrophages subpopulations count, location and gene expressions at day 8 post**
679 **PRRSV-Lena or swIAV-H1N2 infections.**

680 A) Lymph node cells were stained for macrophage subpopulations phenotyping, analyzed (see **Sup.**
681 **Fig. 2**) and sorted using a flow cytometer cell sorter. Absolute macrophage subpopulations numbers,
682 as calculated from absolute total cell number count (**Supp. Fig.1A**) and flow cytometry macrophage

683 subpopulations percentages. B) & C) Microscopic analysis of whole LN (see **Sup. Fig. 5**) stained for
684 Pax5 (green), CD163 (red) and CD169 (blue). B) representative images of trachea bronchial LN from
685 each infectious condition. Black arrows with white outlines and white arrows with black outlines
686 indicate CD163^{pos}/CD169^{neg} MΦ in the T cell areas and trabeculae, respectively. C) LN macrophage
687 subpopulations count in T cell areas and trabeculae as defined in Material and Methods. D) CCL2 and
688 PD-L1 relative transcriptomic expressions of sorted macrophage subpopulations. CD163^{pos}/CD169^{neg}:
689 CD163^{pos}/CD169^{neg} macrophages, LN: lymph node, effMΦ: efferent macrophages, pfMΦ:
690 perifollicular macrophages. Wilcoxon matched-pairs signed rank test, *p*-value: *<0.05, **<0.01,
691 ***<0.001.
692

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694

Table 1 : Antibodies used for flow cytometry and fluorescent microscopy analysis

Ab	Clone	Species/Isotype	Fluorophore	Supplier
Primary Abs				
anti-CD169	1F1 CR4	mIgG2a	none	J. Dominguez, INIA
anti-CD163	2A100	mIgG1	PE	Serotec
anti-CD163	2A100	mIgG1	none	Serotec
anti-CD21	B-ly4	mIgG1	FITC	BD-Pharma
anti-CD21	B-ly4	mIgG1	none	BD-Pharma
anti-IgM	PG145A	mIgM	none	MAC-WSU
anti-CD172a	74-22-15a	mIgG2b	none	MAC-WSU
anti-Pax5	1H9	rat	none	Thermofisher
anti-Bcl6	K112-91	mIgG1	none	BD-Pharma
Secondary Abs				
anti-mIgM		goat	Alexa-647	Invitrogen
anti-mIgG2b		goat	APC-Cy7	Invitrogen
anti-mIgG2a		goat	PE-Cy7	Invitrogen
anti-mIgG2a		goat	Alexa-647	Invitrogen
anti-rIgG		goat	Alexa-488	Invitrogen
anti-rIgG		goat	Alexa-647	Invitrogen
anti-mIgG1		goat	Alexa-555	Invitrogen

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698 **Table 2: RT-qPCR primers**

Gene	Forward	Reverse	Eff	Size	Tm	Ref
RPS24	AAGGAACGCAAGAACAGAATGAA	TTTGCCAGCACCAACGTTG	0.95	62	60	(24)
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCCTCCGCTTACCG	0.90	147	60	(24)
MAFB	TGCGTTCTTTAGACCAATATGTTATGT	CACCAATAAATCGCCCGCTAT	0.99	71	60	(25)
CSF1R	CGGTATGTGCCGTGTTTTG	CGATGCGTGAGCAATGTCAG	0.96	86	60	(24)
CCR2	ACACGCTTCCCGTTCA	CCCTTGATATTCATTGTAAGCAGAGA	0.88	70	59	(26)
IL6	CTGCTTCTGGTGATGGCTACTG	GGCATCACCTTTGGCATCTT	0.94	69	60	(8)
PDL1	GTGGAAAAATGTGGCAGCCG	TGCTTAGCCCTGACGAACTC	0.91	140	58	(27)
CXCL13	ATCTCTGCTTCTCGTGCTG	ACTTCTCTGGTTGGACATCC	1.03	180	60	designed
CCL2	CGGCCAGGACCCATCAG	GAGGGCTGCAGAGACCTTCA	0.86	72	60	designed
TopoIIA	TCAGCCTGGCCTTTAGCAA	TCGTTGCCGTCTATCTCCAT	0.92	67	59	(19)
AID	AGAAGTTTCAAAGCCTGGGAG	TCAACCTCATACAGGGGCAA		92	57	designed
Bcl6	CGAGAAGTGTAACCTGCATTTCC	CGTGCTTCTGGCGCAAGT	0.91	64	60	(19)
Blimp1	TGAAACTCCACAAGCGCCTAC	CCTTCAGATGGACCTTGAGGC	0.90	101	62	designed
CXCR5	CTCTGCAAGACTGTGATAGC	TGGTTGCACAGGTGATATGG	0.94	157	61	designed
swIAVH1N2	AGATGAGTCTTCTAACCGAGGTCG	TGCAAAAAATCTTCAAGTCTCTG		100	60	9
swIAVH1N2 probe	6FAM_TCAGGCCCCCTCAAAGCCGA-TAMRA					
PRRSV Lena	AGAACCAGCGCCAATTCAGA	TCTTTTTCGCCTGTCCTCCC		166	60	8
PRRSV Lena probe	6FAM-AAACACAGCTCCAATGGGGAAATGGC-TAMRA					

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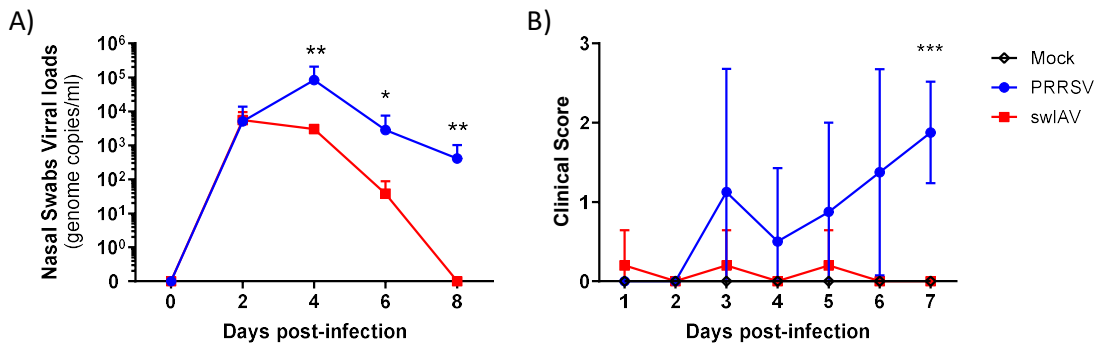


Figure 1: PRRSV Lena and swIAV H1N2 infections present contrasting virus shedding and clinical signs.

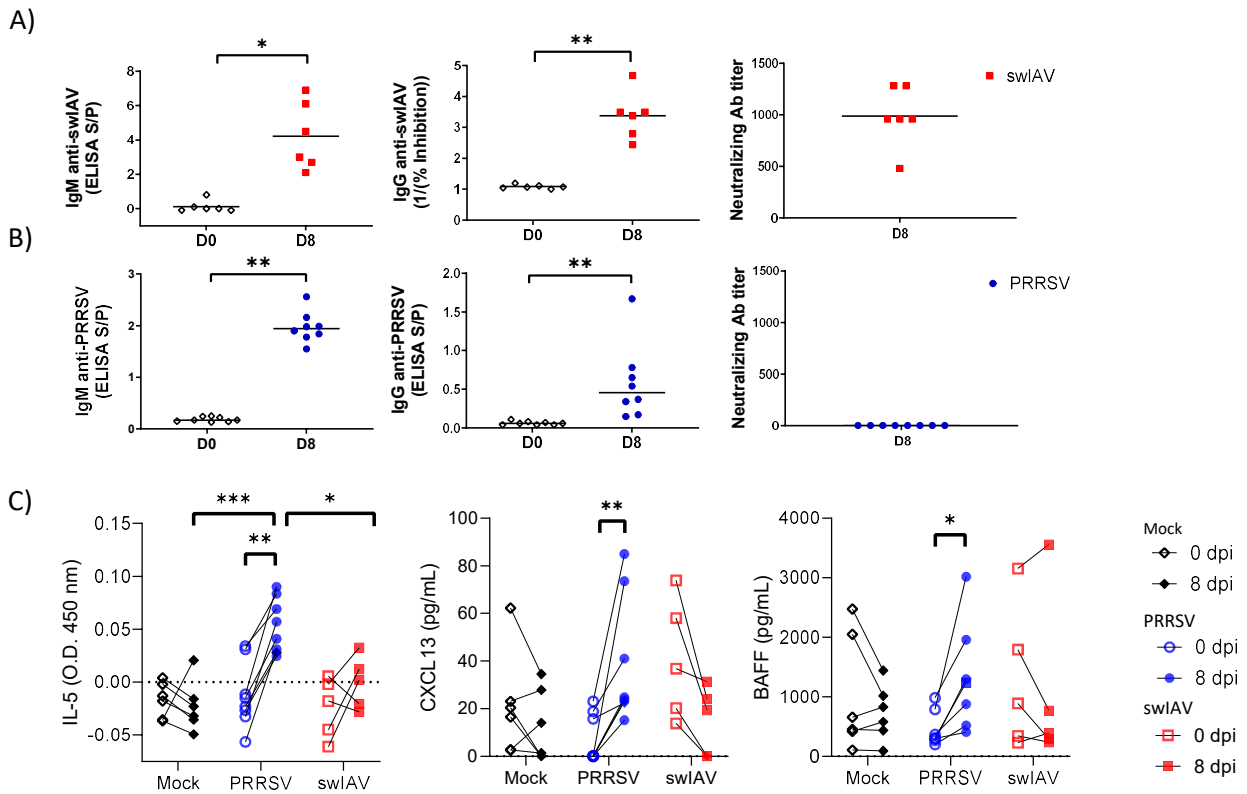


Figure 2: Serum, virus-specific IgM, IgG and neutralizing antibodies and B cell maturation-related cytokines and chemokines concentrations.

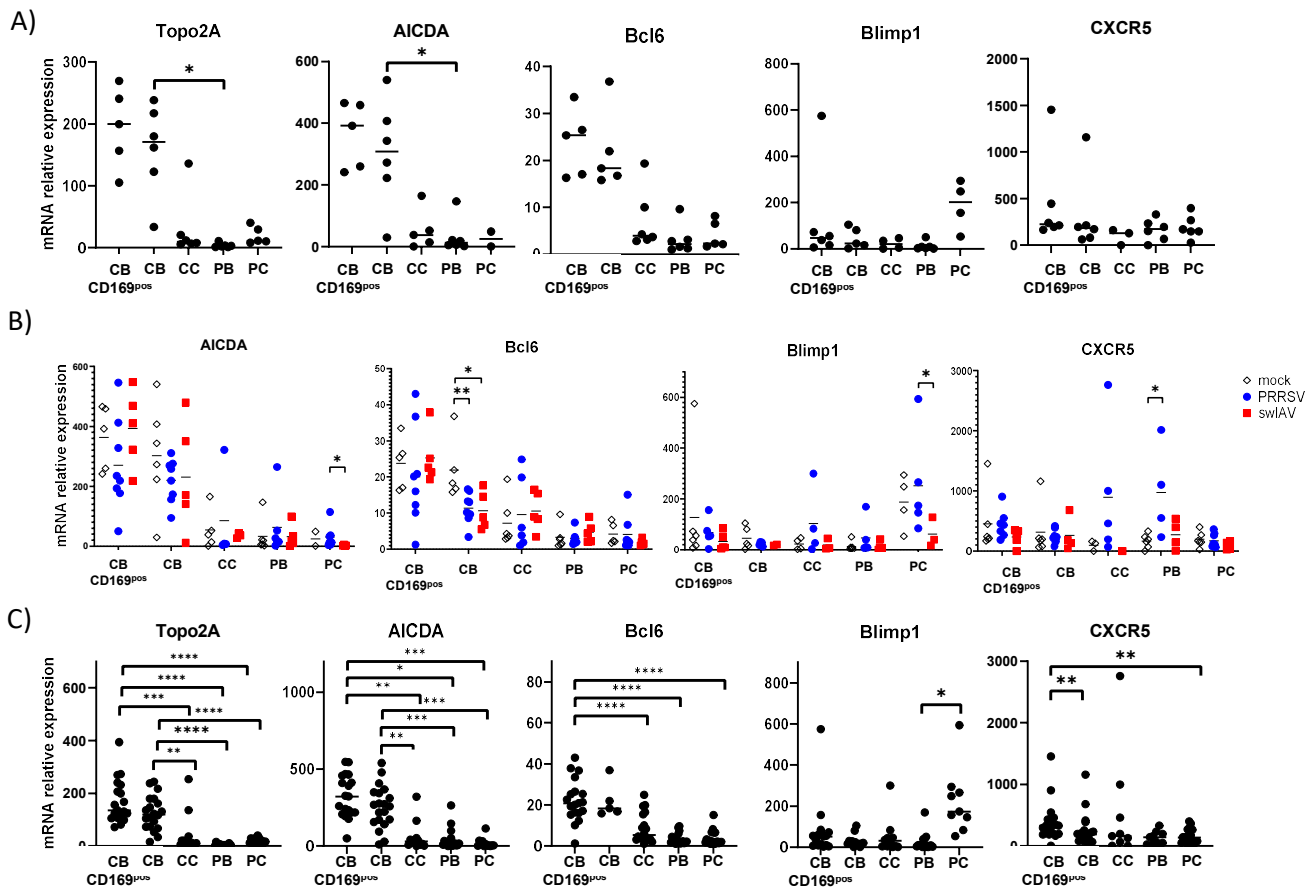


Figure 3: B cells maturation stage specific gene expressions

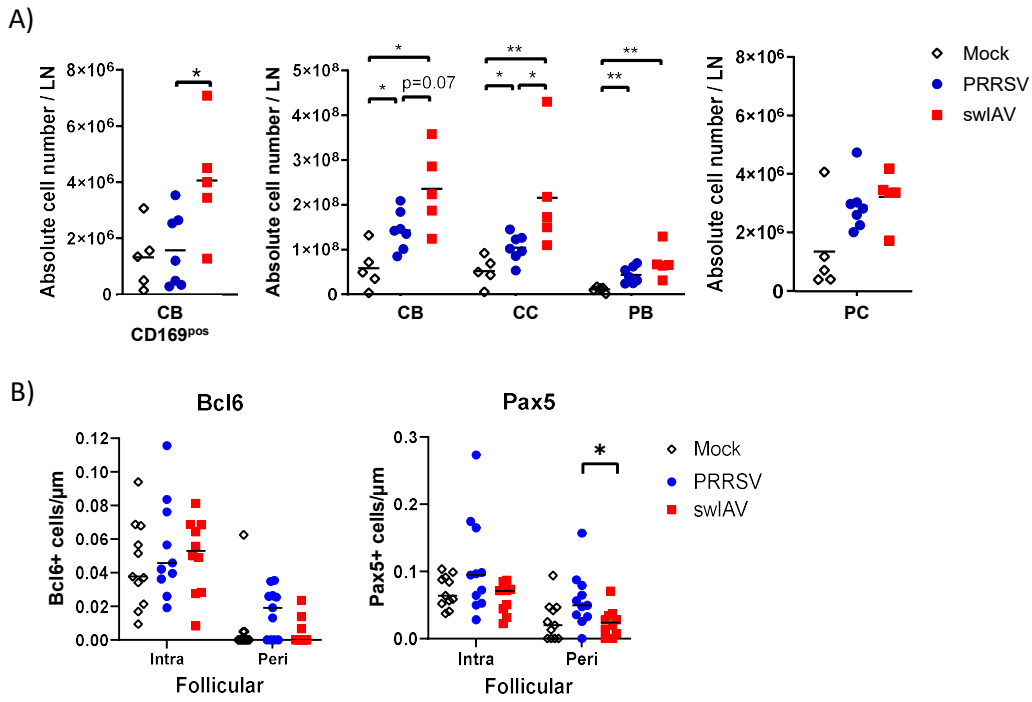


Figure 4: LN maturing B cells count and location at day 8 post PRRSV-Lena and swIAV-H1N2 infections.

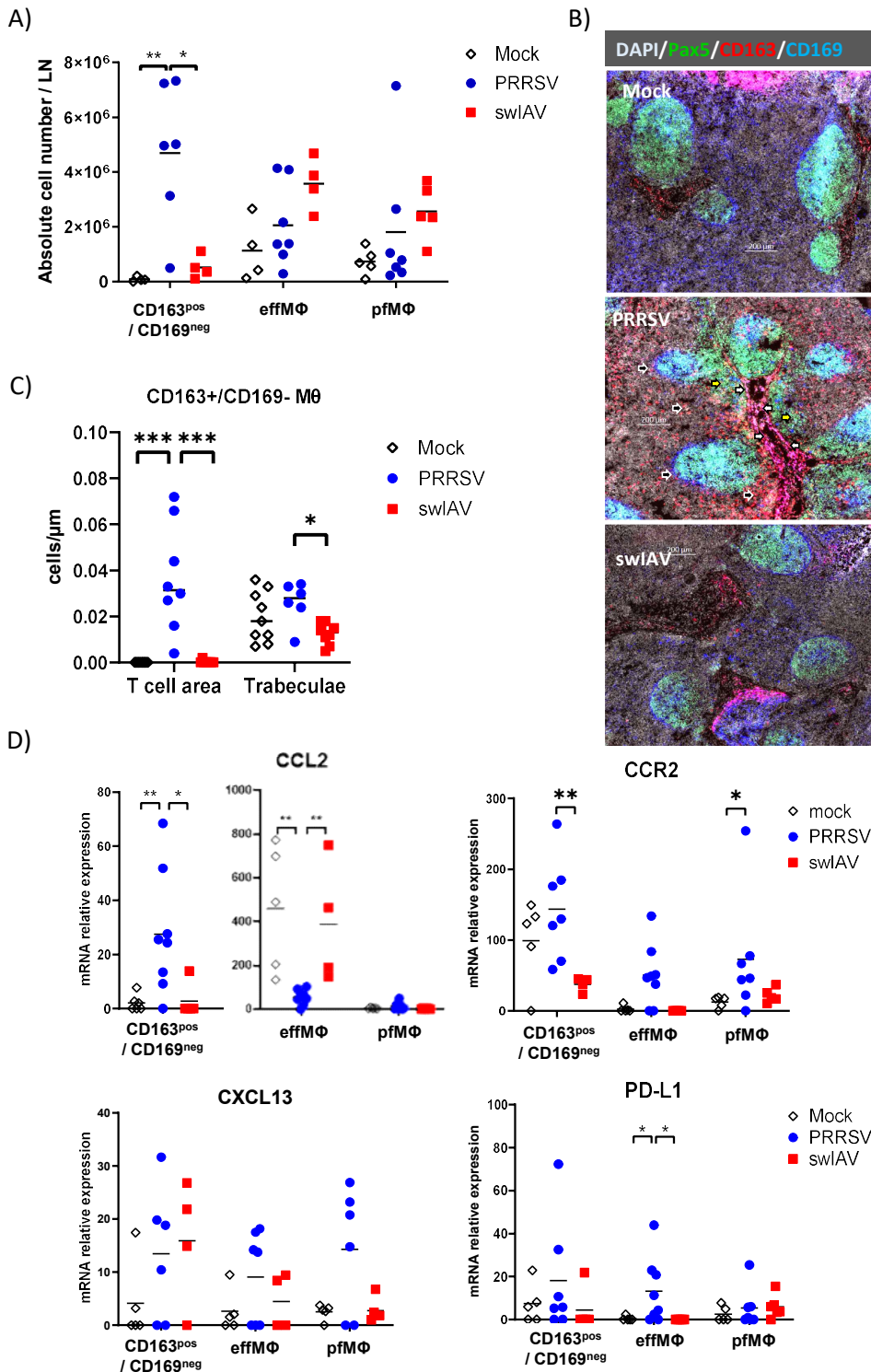


Figure 5: Lymph node macrophages subpopulations count, location and gene expressions at day 8 post PRRSV-Lena and swIAV-H1N2 infections.