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1 **Combined regulation of pro-inflammatory cytokines production**  
2 **by STAT3 and STAT5 in a model of *B. pertussis* infection of**  
3 **alveolar macrophages**

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13

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20 **STAT proteins<sub>5</sub>**

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24

25 **Abstract**

26 *Bordetella pertussis* is a highly contagious respiratory pathogen responsible for whooping-  
27 cough or pertussis. Despite high vaccination coverage worldwide, this gram-negative  
28 bacterium continues to spread among the population. *B. pertussis* is transmitted by aerosol  
29 droplets from an infected individual to a new host and will colonize its upper respiratory tract.  
30 Alveolar macrophages (AMs) are effector cells of the innate immune system that phagocyte  
31 *B. pertussis* and secrete both pro-inflammatory and antimicrobial mediators in the lungs.  
32 However, understanding their role in *B. pertussis* pathogenesis at the molecular level is  
33 hampered by the limited number of primary AMs that can be collected *in vivo*. In our study,  
34 we used self-renewing, non-transformed cells, called Max Planck Institute (MPI) cells, which  
35 are phenotypically and functionally very close to pulmonary AMs in order to decipher the  
36 regulation of innate response induced by *B. pertussis* infection. Using optimized infection  
37 conditions, we characterized the entry and the clearance of *B. pertussis* within MPI  
38 macrophages. We showed that under these conditions, MPI cells exhibit a pro-inflammatory  
39 phenotype with the production of TNF, IL-1 $\beta$ , I-L6 and MIP-2 $\alpha$ , similarly to primary AMs  
40 purified from broncho-alveolar fluids of mice. We explored the yet uncharacterized role of the  
41 signal transduction activator of transcription (STAT) proteins family in the innate immune  
42 response to *B. pertussis* infection and showed for the first time the parallel regulation of pro-  
43 inflammatory cytokines by STAT3 and STAT5 in MPI macrophages infected by *B. pertussis*.

44

45

46 **Introduction**

47 Whooping cough or pertussis is a highly transmissible respiratory disease, responsible for  
48 more than 19 million cases worldwide and 117,000 deaths in children under 5 years old  
49 reported in 2019 (1). Despite being a vaccine-preventable disease, whooping cough still  
50 represents a major health problem due to an increasing incidence which has been observed  
51 since the early 2010s (2). The inability of current acellular pertussis vaccines to prevent nasal  
52 infection and transmission and to induce long-lasting protection are the main reasons for the  
53 resurgence of pertussis (3). Whooping cough is caused by the human-restricted pathogen  
54 *Bordetella pertussis*, a gram-negative bacterium transmitted by aerosol droplets (4). Once  
55 inhaled from an infected person, *B. pertussis* will express adhesins and toxins which will  
56 promote the colonization of the respiratory tract of the newly infected host and will induce  
57 damages in the first line of defense which is the airway epithelium. Resident and recruited  
58 innate immune cells such as alveolar macrophages and dendritic cells will be among the first  
59 immune cells to sense and to respond to *B. pertussis* infection in the lungs (5).

60 Alveolar macrophages (AMs) are the major macrophage population in the lungs (6). Their  
61 role during *B. pertussis* pathogenesis is twofold. These professional phagocytic cells play an  
62 important role in controlling infection, since AM depletion in mice promotes *B. pertussis*  
63 infection (7). This mechanism involves a Myd88-Adaptator-Like protein (MAL) dependent-  
64 pathway and the early induction of pro-inflammatory cytokines and chemokines (8). As well  
65 as contributing to protective immunity, AMs may also provide a niche for prolonged *B.*  
66 *pertussis* survival. Indeed, *B. pertussis* have been found inside AMs of infected infants and  
67 children (9,10). Similarly, intracellular survival was observed in mouse models (7,11) as well  
68 as *in vitro* using human and mouse macrophages (8,12).

69 Macrophages are a heterogeneous cell population which differ phenotypically and  
70 functionally depending on their origin and environment (13). Nevertheless, most of the  
71 current knowledge on macrophage responses to *B. pertussis* infection were extrapolated from  
72 data obtained using cells derived from human mononuclear cells (14), monocytic cell lines  
73 such as the THP1 (15,16), or bone-marrow derived macrophages (8). Indeed, studying  
74 interactions between pathogens and alveolar macrophages is hampered by the limited number  
75 of AMs which can be obtained from naïve mice. In the present study, we used the recently  
76 described Max Planck Institute (MPI) cells to study the alveolar macrophage response to *B.*  
77 *pertussis* infection. MPI cells present phenotypical and immunological similarities with  
78 primary murine alveolar macrophages, such as the CD11c expression, low levels of CD14 and  
79 the production of pro-inflammatory cytokines (17). Those cells have been shown to be a  
80 valuable model to study the host-pathogen interaction, such as with *P. aeruginosa* (18), *M.*  
81 *tuberculosis* (19), and more recently with *S. pneumoniae* (20). Here we set up an optimized  
82 protocol for infection of MPI cells by *B. pertussis* and characterized the process of bacterial  
83 internalization and clearance. We compared the production of pro-inflammatory cytokines  
84 and chemokines by infected MPI cells and primary AMs isolated from broncho-alveolar  
85 lavage fluids of mice. Using this valuable cellular model, we identified an original mechanism  
86 of regulation of pro-inflammatory cytokines expression by *B. pertussis* involving the family  
87 of STAT (signal transduction activator of transcription) proteins. Our results showed that the  
88 combined STAT3 and STAT5 inhibition resulted in high level of pro-inflammatory cytokine  
89 production by MPI macrophages in response to *B. pertussis* infection.

90

91

## 92 **Materials and Methods**

### 93 ***Bordetella pertussis* strain and growth conditions.**

94 Gentamicin-resistant derivative of *B. pertussis* B1917 bacteria (21,22) were grown for 36h at  
95 37 °C on Bordet-Gengou (BG) agar (Difco, Detroit, MI) supplemented with 1% glycerol,  
96 10% of Sheep Defibrinated Blood and 10 µg/ml gentamicin (Sigma-Aldrich). The bacteria  
97 were then scrapped off the plates and suspended in RPMI medium at the desired optical  
98 density.

99

### 100 **MPI model**

101 Max Plank Institute (MPI) macrophages are self-renewing, non-transformed, GM-CSF  
102 dependent murine cells, immunologically and phenotypically similar to murine alveolar  
103 macrophages (17). MPI were routinely cultivated in non-treated T-75 flasks (Sarstedt) at 37  
104 °C, 5% CO<sub>2</sub> in RPMI 1640-Glutamax medium (Gibco), supplemented with 10% Foetal Bovine  
105 Serum (Eurobio) and 30 ng/ml GM-CSF (Miltenyi).

106 For infection experiment, adherent cells were gently detached with Versene (Gibco) for 5  
107 minutes at 4 °C, recovered by centrifugation. and then seeded in 6 or 96-well plates in  
108 RPMI1640-Glutamax medium + 10% FBS, complemented or not with 15 ng/ml GM-CSF.

### 109 **Isolation of primary murine alveolar macrophages**

110 8-week old C57BL/6 mice were purchased from Charles River, Laboratories, France. All  
111 mice were maintained at the Institut Pasteur de Lille BSL-2 animal facility. Animal  
112 experiments were carried out according to the guidelines of the French Ministry of Research  
113 on animal experiments and with institutional regulations and ethical guidelines (B59-350009,  
114 Institut Pasteur de Lille, France). The protocols were approved by the Ethical Committees of  
115 the Region Nord-Pas-de-Calais and the Ministry of Research (agreement number APAFIS #  
116 201603311654342\_v2). To recover broncho-alveolar lavage fluids (BALFs), mice were  
117 euthanized by intraperitoneal injection of pentobarbital (Euthasol, TVM Lab). A canule was  
118 inserted in the trachea and BALFs were recovered by lavages with 800 µl of a pre-warmed  
119 solution of PBS (Gibco), 2% FBS and 2 mM EDTA and centrifuged at 300g, at 4°C for 5  
120 min. Erythrocytes were lysed using ACK Lysing Buffer (Gibco). Cell pellets were  
121 resuspended in RPMI-1640-Glutamax, 10% FBS, 1 mM Pyruvate (Gibco) and GM-CSF (15  
122 ng/ml) and were seeded in 96-well tissue-culture treated plates (CytoOne) at a final  
123 concentration of  $2 \times 10^5$  cells/well. Purity of alveolar macrophages was assessed by Facs  
124 analysis and was shown to be higher than 90% (data not shown).

### 125 **Macrophages infection with *B. pertussis***

126 Macrophages were seeded in 6 or 96-well plates (CytoOne) at a concentration of  $2 \times 10^5$   
127 cells/well, with or without GM-CSF (15 ng/ml) and incubated at 37°C under 5% CO<sub>2</sub>. After  
128 24h of incubation, the macrophages were infected with *B. pertussis* for 30 min, 1h or 2h at a  
129 multiplicity of infection (MOI) of 10, 50 or 100 bacteria/cell. The end of the time contact  
130 corresponds to T0. Non-adherent *B. pertussis* were removed by two successive washes with  
131 PBS and remaining extracellular bacteria were killed by 1h treatment with 100µg/ml  
132 polymyxin B (Euromedex) diluted in the culture medium. Cellular viability was assessed at  
133 different time points after infection using the MTT assay (Cell Kit Proliferation I, Roche) and  
134 measurement of the Optical Density (OD) on a plate reader at  $\lambda = 570\text{nm}$ . The results are  
135 expressed as the ratio of the  $(\text{OD}_{\text{stimulated cells}}/\text{OD}_{\text{unstimulated cells}}) * 100$ . To evaluate the  
136 intracellular bacterial loads, macrophages were lysed at indicated times with 0,1% saponin  
137 (Sigma) and serial dilutions of the lysates were plated on BG agar medium. After 5 days of

138 incubation at 37°C, the Colony Forming Units (CFU) were counted on the plates. Intracellular  
139 survival is calculated with the ratio of  $(CFU_{\text{Time of interest}}/CFU_{T0}) * 100$ . In the experiments  
140 aiming to assess the impact of phagocytosis, MPI cells were pre-treated with 10µM of  
141 Cytochalasin D (Sigma-Aldrich) 30 min prior to *B. pertussis* infection.

#### 142 **Transmission electron microscopy**

143 After 2h and 4h of contact with *B. pertussis*, MPI cells were fixed with 1% glutaraldehyde in  
144 0.1 M sodium cacodylate pH 6.8 buffer, at 4°C overnight. Infected macrophages were post-  
145 fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide then with 1% uranyl  
146 acetate, both in distilled water at room temperature in the dark, for 1h. After washing, samples  
147 were dehydrated with increasing ethanol-concentration solutions. Samples were finally  
148 infiltrated with epoxy resin and cured at 60°C for 24h. Sections of 70-80 nm thickness  
149 deposited on formvar-coated grids were observed at 80 kV with a Hitachi H7500 TEM  
150 (Milexia, France), and images were acquired with a 1 Mpixel digital camera from AMT  
151 (Milexia, France).

#### 152 **Immunoblot**

153 MPI cells were lysed in 0.1% Triton X-100 (Sigma), 5mM EDTA, 50 mM NaCl, 20 mM Tris-  
154 HCl pH 7,4 and 1% of HALT protease/phosphatase inhibitor (Thermofisher) and the  
155 suspension was clarified by centrifugation. Protein concentration was determined using Pierce  
156 BCA Protein Assay Kit (Thermoscientific). Samples were boiled at 95°C for 10 min in  
157 Laemmli buffer containing 1M of dithiothreitol and proteins were separated on 4-15% Mini-  
158 protean TGX Gel (Bio-rad). Proteins were transferred to nitrocellulose membranes  
159 (Thermoscientific) which were blocked in PBS-Tween 0,1% containing 5% of non-fat milk  
160 for 1h and incubated overnight at 4°C with the following primary antibodies from Cell  
161 Signaling Technology: anti-β-actin (clone 8H10D10, dilution 1:1000), anti-pSTAT3 Tyr705  
162 (clone 3E2, dilution 1:1000), anti-pSTAT3 Ser727 (ref 9134, dilution 1:1000), anti-pSTAT5  
163 (clone C71E5 dilution 1:1000) anti-STAT3 (clone 124H6, dilution 1:1000) and anti-STAT5  
164 (clone D2O6Y, dilution 1:1000). The membrane was then incubated with the appropriate  
165 HRP-conjugated secondary antibodies for 1h30 at room temperature. Revelation was done  
166 using SuperSignal West Femto Maximum Sensitive Substrate kit (Thermofisher) and images  
167 were acquired on Amersham Imager 600. Band intensity was quantified using ImageJ  
168 software and normalized to loading control β-actin. Relative expression for the condition of  
169 interest was then normalized to the one obtained for GM-CSF non-infected cells, which give  
170 the fold-protein expression.

171

#### 172 **Flow-Cytometry**

173 To assess the rate of macrophages infected with *B. pertussis*,  $10^9$  bacteria were resuspended in  
174 1 ml PBS containing 5 mM EDTA and incubated with 15µg/ml carboxyfluorescein diacetate  
175 succinimidyl ester (CFSE) (BD Horizon) at 37°C under agitation for 20 min. The CFSE  
176 fluorescence intensity was measured on xxx FACS to quantify the percentage of MPI infected  
177 cell. The bacteria were then diluted to the desired concentration prior to the infection.

178 For MPI cells phenotyping, cells were washed in PBS containing 3% FBS and 1 mg/ml  
179 sodium azide (Sigma). Cells were then incubated with Fc Block (BD Pharmingen, dilution  
180 1:200), followed by staining with anti-SiglecF APC (Biolegend, clone S1700L dilution  
181 1:400), anti-CD11c BV711 (Biolegend, clone N418 dilution 1:400), anti-CD11b BV605 (BD  
182 Horizon, clone M1/70 dilution:1:400), anti-CD16/CD32 FITC (BD Pharmingen, clone 2.4 G2  
183 dilution 1:200), anti-Ly6G PE-CF594 (BD Horizon, clone 1A8 dilution 1:400) and anti-F4/80  
184 PE (eBioscience, clone BM8 dilution 1:300). Cells were washed and fixed in  
185 Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Fluorescence-activated cell

186 sorting samples were acquired on an Attune NxT using the Attune NxT Software  
187 (ThermoFisher scientific) and analyzed using the FlowJo v10 Software.  
188 For cell cycle analysis, macrophages were harvested and fixed overnight in 70% ethanol at -  
189 20 °C. Cells were then washed in PBS and stained with 10 µg/mL propidium iodide (Sigma)  
190 containing RNase A (200 µg/mL; ThermoScientific) at 37 °C for 15 min in the dark. Cellular  
191 DNA content was measured from at least 10000 events per sample by LSR Fortessa  
192 (Beckman Coulter) and cell cycle distribution was analyzed with FlowJo v10 software with  
193 the Dean-Jett-Fox univariate model.

194

### 195 **Cytokine analysis**

196 To determine cytokine secretion level, cell culture supernatants were collected 24h after  
197 infection and analysed by enzyme-linked immunosorbent assay (ELISA) and Luminex  
198 technology according to the manufacturer's instructions. The following cytokines were  
199 assessed by ELISA : TNF (Mouse TNF ELISA Set II, BD OptEIA), IL-6 (Mouse IL-6 ELISA  
200 Set, BD OptEIA), IL-1β (Mouse IL-1β Uncoated ELISA Kit, Invitrogen) and MIP-2α  
201 (DuoSet Mouse CXCL2/MIP-2, R&D Systems). When indicated, the production of cytokines  
202 was measured by Luminex technology using the following Milliplex Mouse magnetic bead  
203 panel (Merck, Millipore): TNF, IL-6, IL-1β, MIP-2α, IL12p40, IL-5, IL-10 and IL-22.

204 To measure the level of gene expression, total RNA was extracted and purified using the  
205 NucleoSpin kit (Macherey-Nagel). RNA quantification and RIN number were determined  
206 respectively with Nanodrop 2000c (ThermoFisher) and the Bioanalyzer (Agilent). The RNAs  
207 were selected with a RIN number > 8. Reverse Transcription was then performed on 300 ng  
208 of purified RNA with the Verso cDNA Synthesis kit (Thermo Scientific). The obtained cDNA  
209 was mixed with the No ROX SYBR 2X MasterMix blue dTTP (Eurogentec-Takyo) and with  
210 specific primers for the genes of interest (GOI) (Supplementary Table 1) obtained from  
211 Eurogentec. qPCR was then realized with a LightCycler480. The Ct of all GOIs were  
212 subtracted to the Ct of the macrophage housekeeping gene, Syntaxin-5 gene (*stx5a*) (Tanaka  
213 et al, 2017). The  $2^{-\Delta\Delta Ct}$  formula was then applied and considered as the fold-gene expression  
214 relative to the control condition.

### 215 **Statistical Analysis**

216 Multiple independent groups were analyzed using first a Kruskal-Wallis test then a Conover  
217 post-hoc test. The Mann-Whitney U test was used to compare differences between two  
218 independent groups. If the p-value was 0.05 or less, the results were considered statistically  
219 significant. All the graphs, calculations and statistical analyses were performed by using  
220 GraphPad Prism software 141 version 9.3.1 (GraphPad Software, San Diego, CA, USA) and  
221 R software version 4.1.1 (R 142 Foundation for Statistical Computing, Vienna, Austria).

222

223

224

## 225 **Results**

226

### 227 **Optimization of *B. pertussis* infection in the MPI macrophage model**

228 We first assessed MPI cell response to *B. pertussis* contact using as readout the secretion of  
229 TNF, a pro-inflammatory cytokine produced by *B. pertussis*-infected alveolar macrophages  
230 (8). MPI cells were cultured for 30 min with B1917, a virulent strain representative of current  
231 circulating *B. pertussis* bacteria (21). We compared increasing doses of B1917 corresponding  
232 to 10, 50 and 100 bacteria/cell. We showed that a multiplicity of infection (MOI) of 50 is

233 required to induce significant secretion of TNF by MPI macrophages, and that the level of  
234 secreted TNF is increased at a MOI of 100 (Figure 1A). In parallel, we measured the impact  
235 of *B. pertussis* on MPI viability. Using the MTT assay, we observed a MOI-dependent  
236 increased cellular metabolic activity, indicating that B1917 stimulates MPI proliferation  
237 (Figure 1B). No significant difference was detected between a MOI of 50 and 100 suggesting  
238 that the maximum proliferation level was already reached with the MOI of 50.

239 *B. pertussis* expresses several adhesins which will interact with receptors at the surface of  
240 macrophages to allow bacterial adherence (23–26). We determined the optimal time contact  
241 between *B. pertussis* and MPI macrophages to induce a significant TNF response. MPI cells  
242 were in contact with B1917 at MOI 50 for 30 min, 1h, or 2h before killing extracellular  
243 bacteria with polymyxin B. One day after infection, significant secretion of TNF was  
244 measured in cell culture supernatants in all conditions with a peak response induced after 1h  
245 of bacterium-cell contact (Fig. 1C). MPI viability was not affected even after the longest time-  
246 contact with *B. pertussis* (data not shown). Taken together, these data indicate that the  
247 optimized protocol of infection of MPI macrophages with *B. pertussis* strain B1917 requires a  
248 contact time of 1h at a MOI of 50.

### 249 **Internalization and clearance of *B. pertussis* by MPI macrophages**

250 Using transmission electron microscopy, we observed that interaction of *B. pertussis* with  
251 MPI induces cellular pseudopods extension which surround bacteria, leading to internalization  
252 of *B. pertussis* inside macrophages (figure 2A). We measured the internalization efficiency of  
253 *B. pertussis* by MPI using the optimal conditions determined previously. Quantitative  
254 assessment by FACS analysis showed that in these conditions, more than 90% of MPI were  
255 infected with *B. pertussis* (Fig 2B).

256 To assess the impact of *B. pertussis* internalization on our readout which is TNF secretion, we  
257 treated MPI with cytochalasin D, an inhibitor of actin polymerization and phagocytosis, prior  
258 to infection. In these experimental conditions, a reduction in bacterial internalization rate of  
259 approximately 40% was observed in MPI treated with cytochalasin D leading to a significant  
260 decrease in TNF secretion after infection (Figure 3A and 3B). MPI viability was not affected  
261 by cytochalasin D treatment (data not shown).

262 We observed by transmission electron microscopy that following internalization, *B. pertussis*-  
263 containing phagosome fuses with lysosome to form a phagolysosome (Figure 4A). We  
264 monitored *B. pertussis* intracellular survival inside MPI cells and showed that *B. pertussis* is  
265 rapidly cleared by MPI macrophages, with less than 10% live bacteria recovered 24 hours  
266 after phagocytosis (Figure 4B). Only few live bacteria were recovered 96 hours after infection  
267 (<10 CFU/million of MPI cells).

268

### 269 **MPI cells unlike primary alveolar macrophages express high level of pro-inflammatory 270 cytokines but no IL-22 in response to *B. pertussis* infection**

271 We compared- after infection with *B. pertussis*, the levels of cytokines produced by the MPI  
272 by primary alveolar macrophages (AMs) purified from broncho-alveolar lavages of C57BL/6  
273 mice. TNF secretion level was relatively higher in primary AMs infected with *B. pertussis*  
274 compared to infected-MPI macrophages even though the difference was not significant  
275 (Figure 5A). In contrast, MPI cells secreted significantly higher amount of the pro-  
276 inflammatory cytokines IL-1 $\beta$  and IL-6 as well as the chemokine MIP-2 $\alpha$  (macrophage  
277 inflammatory protein 2 $\alpha$ ) involved in the recruitment of immune cells, than primary AMs in  
278 response to *B. pertussis* infection (Figure 5B-D). However, and in contrast to primary AMs  
279 (27), infected-MPI cells did not secrete IL-22 (Figure 5E). No secretion of IL12p40, IL-5 nor  
280 IL-10 was detected after *B. pertussis* infection of MPI or primary AMs (data not shown).

281



282 **Pro-inflammatory cytokines are produced by *B. pertussis*-infected MPI macrophages in**  
283 **the absence of GM-CSF-induced STAT5 activation**

284 Regulation of signal transduction activator of transcription (STAT) protein family is critical  
285 for the induction of pro- or anti-inflammatory responses (28). Among the STAT family,  
286 STAT5 was shown to be activated by GM-CSF and to be essential for MPI pro-inflammatory  
287 phenotype (17). We therefore assessed the role of STAT5 in the induction of pro-  
288 inflammatory cytokine secretion in response to *B. pertussis* infection. Unexpectedly, we  
289 found that STAT5 activation, measured by its phosphorylation level, in MPI macrophages is  
290 downregulated after *B. pertussis* infection (Figure 6A and 6B). In order to confirm that the  
291 production of pro-inflammatory cytokines is independent of GM-CSF-induced STAT5  
292 activation, we cultured MPI cells in medium depleted of GM-CSF before *B. pertussis*  
293 infection. As expected, removal of GM-CSF from MPI cells medium resulted in the absence  
294 of activation of STAT-5 in uninfected and *B. pertussis*-infected MPI lysates (Figure 6A and  
295 6B). GM-CSF starvation being known to induce G0/G1 cell cycle arrest which could impact  
296 cellular properties and lead to comparison biases, we ensured that *B. pertussis* infection was  
297 able to induce cell cycle progression despite the removal of GM-CSF from culture medium.  
298 FACS analysis of MPI cells cultured in GM-CSF-depleted or complete medium showed that *B.*  
299 *pertussis* infection induced re-entry into the cell cycle as highlighted by an increased number  
300 of MPI-infected cells in G2/M phases in both conditions 24h after infection (Table 1).  
301 Moreover, *B. pertussis* killing inside infected-MPI cells cultured with GM-CSF was  
302 significantly less efficient than in MPI macrophages grown in a medium without GM-CSF  
303 (Figure 7). Cellular viability of infected-MPI cells was not affected by the presence or not of  
304 GM-CSF (data not shown). Finally, MPI cell phenotypes analysed by FACS after 24 hours of  
305 GM-CSF starvation showed lower level of CD11c and CD32 compared to MPI cells cultured  
306 in medium with GM-CSF while no difference was observed for the other markers studied  
307 (SiglecF, CD11b, F4/80, Ly6G) (Figure S1). The levels of expression and secretion of  
308 cytokines were then measured in *B. pertussis*-infected MPI cells cultured with or without  
309 GM-CSF. We showed that in response to *B. pertussis*, higher transcript levels of TNF, IL-1 $\beta$ ,  
310 IL-6 and MIP-2 $\alpha$  were detected 6 hours after infection when MPI were cultured without GM-  
311 CSF and therefore when STAT5 is inactivated (Figure 8A). In addition, 24h after *B. pertussis*  
312 infection, significantly higher levels of TNF, IL-1 $\beta$ , IL-6 and MIP-2 $\alpha$  were secreted in  
313 supernatants of MPI cells cultured without GM-CSF (Figure 8B).

314  
315 **Combined downregulation of STAT5 and Tyr705 STAT3 phosphorylation, but not**  
316 **Ser727 STAT3, by *B. pertussis* infection leads to pro-inflammatory cytokine production**  
317 **by MPI macrophages**

318 We hypothesized that the surprisingly higher pro-inflammatory cytokines production  
319 observed in the absence of GM-CSF-induced STAT5 activation might be explained by the  
320 parallel downregulation of STAT3. Indeed, it was shown in a model of intestinal myeloid  
321 cells and upon *Salmonella typhimurium* exposure that combined STAT3 and STAT5  
322 inhibition resulted in high level of pro-inflammatory cytokine production (29). We therefore  
323 analysed STAT3 activation in *B. pertussis*-infected MPI cells cultured in medium without  
324 GM-SCF, conditions where we showed that STAT5 activation was inhibited. We found that  
325 similarly to STAT5, activation of STAT3 measured by the phosphorylation of Tyr705 was  
326 downregulated compared to *B. pertussis*-infected MPI cells cultured in complete medium  
327 (Figure 9A and 9C). STAT3 phosphorylation on Tyr705 induces its translocation to the  
328 nucleus to regulate transcription, while Ser727 phosphorylated STAT3 leads to mitochondrial  
329 metabolic reprogramming (ref). Removal of GM-CSF from MPI cells medium resulted in the  
330 downregulation of Tyr705 but not Ser727 STAT-3 phosphorylation after *B. pertussis*  
331 infection (Figure 9B and 9C).

333 **Discussion**

334 Alveolar macrophages are among the first innate cells to encounter *B. pertussis* when the  
335 bacteria reach the lungs. Their dual role as a potential reservoir of *B. pertussis* and as a key  
336 player in induction of inflammatory and immune responses make them a particularly  
337 attractive type of cells to study *B. pertussis* pathogenesis. Our current understanding of the  
338 molecular mechanisms induced in AMs in response to *B. pertussis* infection is nevertheless  
339 limited due to the constraints in collecting primary AMs which are present in low numbers in  
340 the lungs of naïve individuals. In this paper, we describe the first data obtained with MPI  
341 cells, macrophages phenotypically similar to murine alveolar macrophages (17), in the context  
342 of *B. pertussis* infection. We showed in an optimized infection protocol that MPI cells can  
343 phagocytose and kill intracellular *B. pertussis*. We observed by transmission electron  
344 microscopy that phagosomes containing *B. pertussis* fused with lysosomes, which is a major  
345 driver of bacterial killing (30). This is consistent with the decrease in the number of viable  
346 intracellular bacteria in MPI cells within 24 hours of infection and is in agreement with a  
347 previous study showing colocalization of *B. pertussis* with late endosomal/lysosomal marker  
348 LAMP-1 and with Lysotracker, an acidotropic dye, indicating a lysosomal fusion in  
349 macrophages derived from blood mononuclear cells (31). In contrast to this study, we haven't  
350 detected multiplication of *B. pertussis* inside MPI macrophages and only very few live  
351 bacteria were recovered from infected MPI after 3 days of infection. Macrophages represent a  
352 very heterogenous and specialized group of cells (32) and the difference observed between the  
353 two studies could be related to the distinct features of AMs compared to monocyte- or bone  
354 marrow-derived macrophages (33,34). Interestingly, a recent study suggested that *B. pertussis*  
355 may survive for a longer time inside macrophages in a dormant, non-culturable state (35).  
356 This observation opens a new axis that will have to be further explored in order to understand  
357 the intracellular fate of *B. pertussis* inside alveolar macrophages, which have been described  
358 as potential infectious reservoir, in particular because of their long lifespan (36).  
359 Another characteristic of alveolar macrophages is that they play a central role in the  
360 orchestration of the innate immune response during respiratory infection. In response to *B.*  
361 *pertussis* infection, both MPI macrophages and primary AMs purified from broncho-alveolar  
362 lavages of mice exhibit pro-inflammatory phenotypes with the secretion of TNF, IL-1 $\beta$  and  
363 IL-6. In addition, we showed that both MPI and primary AMs expressed MIP-2 $\alpha$ , also called  
364 CXCL2 (37), involved in the recruitment of neutrophils which play a key role in the clearance  
365 of *B. pertussis* (38,39). IL-22 has also been shown to play an important role in mucosal  
366 response to gram-negative pathogens, as well as reducing lung injury by promoting mucosal  
367 barrier integrity (40). In addition, IL-22 was shown to synergize with IL17, a cytokine which  
368 plays a key role in protective immunity against *B. pertussis* infection (41), to enhance  
369 antimicrobial peptide production and neutrophil recruitment (42). We showed here that  
370 primary AMs produce IL-22 in response to *B. pertussis* infection and may therefore contribute  
371 to the host defense response against *B. pertussis*. MPI cells were previously shown to be  
372 unable to produce IL-10 in response to various stimuli (17). Similarly, we did not detect IL-10  
373 production in response to *B. pertussis* infection of MPI macrophages nor IL-22 which belongs  
374 to the same family of cytokines as IL-10 (43). Regulation of cytokine production is a complex  
375 pathway, which has been surprisingly poorly studied during *B. pertussis* pathogenesis. In this  
376 work, we explored for the first time the role of the signal transduction activator of  
377 transcription (STAT) protein family in the innate immune response to *B. pertussis* infection.  
378 STAT proteins are part of the Janus kinase-signal transducer and activator of transcription  
379 (JAK-STAT) pathway, which is an evolutionary-conserved signalling pathway essential for  
380 the regulation of the innate immune response (44). Briefly, the JAKs are activated by binding

381 of extracellular ligands, such as cytokines or growth factors, to transmembrane receptors.  
382 Activated JAKs then phosphorylate STAT proteins, which are translocated to the cell nucleus  
383 where they will bind to DNA and activate transcription of specific genes (44). Among the 7  
384 members of the STAT family, STAT5 phosphorylation is dependent upon GM-CSF  
385 stimulation of cells and JAK2 activation (45). GM-CSF is critical for lung alveolar  
386 macrophage differentiation, and its presence in the culture medium is required for the  
387 proliferation of MPI cells (17,46). Surprisingly and contrary to a previous study using various  
388 stimuli (17), we showed here that in the absence of GM-CSF in the culture medium, high  
389 levels of pro-inflammatory cytokines were induced by *B. pertussis* infection of MPI cells  
390 suggesting that the induction of pro-inflammatory cytokines by *B. pertussis* is independent of  
391 STAT5 activation. In neutrophils, GM-CSF was shown to induce the specific phosphorylation  
392 of STAT5 but also STAT3 (47). Interestingly, STAT3 has been shown to cooperate with  
393 STAT5 to regulate the balance between pro- and anti-inflammatory cytokines response and  
394 particularly the parallel downregulation of STAT3 and STAT5 resulted in high level of pro-  
395 inflammatory cytokine production (29). Similarly, we showed that STAT5 and also STAT3  
396 phosphorylation on Tyr705 was inhibited in MPI cells cultured in medium depleted in GM-  
397 CSF and infected with *B. pertussis* and that this combined inhibition was associated with an  
398 increase in production of pro-inflammatory cytokines.

399 In summary, our study described the use of a valuable model, called MPI cells, to study the  
400 interaction between *B. pertussis* and alveolar macrophages, sentinel cells of the lungs playing  
401 a key role in innate immune defense against infection. Using this model, we explored for the  
402 first time the role of two proteins from the STAT family in the regulation of pro-inflammatory  
403 cytokine production induced by *B. pertussis* infection of alveolar macrophages. Our study  
404 showed that induction of pro-inflammatory cytokines by *B. pertussis*-infected MPI cells was  
405 linked to the combined inhibition of STAT5 and STAT3 activation in the model of MPI  
406 alveolar macrophages.

407

408

#### 409 **Author contribution**

410 FK, CR and NM conceived and planned the experiments. FK, ZK, VD, SS and EP performed  
411 *in vitro* experiments and processed the data. FK, ASD, and CR contributed to the design of  
412 animal experiments and processed animal samples. FK, SC, CR and NM performed the  
413 statistical analysis and data curation. NB performed the TEM. FK, CR and NM wrote the  
414 manuscript with input from all authors. CR and NM directed the project and contributed to  
415 funding acquisition. All authors provided critical feedback and helped shape the research,  
416 discussed the results and commented on the manuscript.

417

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428

#### 429 **Contribution to the field statement**

430 Alveolar macrophages are innate cells that act as sentinels to trap infectious pathogens  
431 reaching the lungs. Although their role in *B. pertussis* pathogenesis has been shown to be  
432 critical in protective immunity, the molecular mechanisms involved are not yet fully  
433 understood, in particular due to the limited number of cells that can be recovered from a naïve  
434 individual. In this work, we used for the first time a new cellular model, the MPI cells,  
435 phenotypically and functionally very close to alveolar macrophages to explore the regulation  
436 of the production of pro-inflammatory cytokines by infected macrophages. This study sheds  
437 new light on the molecular mechanisms induced by *B. pertussis* inside macrophages to trigger  
438 early immune responses.

439

440

441

## 442 **Figure legends**

443

444 **Figure 1. TNF production by MPI cells infected with *B. pertussis*.** (A) TNF production  
445 24h after infection measured by ELISA from cell culture supernatant of MPI cells incubated  
446 for 0,5h with different MOI of *B. pertussis*. (B) MPI cell viability assessed 24h after infection  
447 using MTT assay. (C) TNF production measured by ELISA from cell culture supernatant of  
448 MPI cells incubated with *B. pertussis* for 0.5, 1 or 2h. Lines represent the median of triplicates  
449 or quadruplicates. Statistical test used: Kruskal-Wallis followed by a Conover's test for  
450 multiple comparisons. Data are representatives of three independent experiments. \*: p-value  
451 <0,05, \*\*: p-value <0,01.

452

453 **Figure 2. Internalization of *B. pertussis* by the MPI cells.** (A) Electron micrograph of a  
454 cross-sectioned MPI cell incubated for 2h with *B. pertussis*. *B. pertussis* is entrapped by  
455 cytoplasmic protrusions synthesized by the cell. Scale bar, 500 nm. (B) Representative Flow  
456 cytometry chart of MPI cells infected with CFSE-stained-*B. pertussis*. Data are  
457 representatives of 3 independent experiments.

458

459 **Figure 3. Impact of *B. pertussis* internalization on TNF response by MPI cells.** (A)  
460 Number of colony-forming unit (CFU) internalized by MPI cells treated or not with  
461 cytochalasin D (CytD). (B) TNF production 24h after *B. pertussis* infection measured by  
462 ELISA from cell culture supernatant of MPI cells treated or not with Cyt D. Lines represent  
463 the median of quadruplicates. Statistical test used: Mann-Whitney. Data are representatives of  
464 3 experiments. \*: p-value < 0,05.

465

466 **Figure 4. Intracellular clearance of *B. pertussis* by MPI cells.** (A) Electron micrograph of a  
467 cross-sectioned MPI cells 4h after *B. pertussis* infection showing the fusion of a *B. pertussis*-  
468 containing phagosome with a lysosome. Scale bar, 500 nm. (B) Kinetics of intracellular  
469 survival of *B. pertussis* inside MPI cells. The bars represent the mean of quadruplicates. Data  
470 are representatives of 3 independent experiments.

471

472 **Figure 5. Cytokine responses of MPI cells and primary murine alveolar macrophages**  
473 **infected with *B. pertussis*.** 24h post-infection, production of TNF, IL-1 $\beta$ , IL-6, MIP-2 $\alpha$  and  
474 IL-22 were measured by the Luminex technology. Lines represent the median of five

475 technical replicates. Statistical test used: Mann-Whitney. Data are representatives of 3  
476 independent experiments. \*: p-value < 0,05.

477 **Figure 6. Inhibition of STAT5 activation in MPI cells infected with *B. pertussis*.** (A)  
478 Phospho-STAT5 (p-STAT5), STAT5 and  $\beta$ -actin levels in lysates of MPI cells infected with  
479 *B. pertussis*. NI : Non-infected cells, BP : *B. pertussis* infected-cells. (B) Fold-protein  
480 expression of STAT5 and p-STAT5 in *B. pertussis*-infected MPI cells cultured in the presence  
481 or not of GM-CSF. Bars represent the median of triplicates. Data are representatives of 3  
482 independent experiments.

483 **Figure 7. *B. pertussis* clearance in MPI cells with and without GM-CSF.** (A) Kinetics of  
484 intracellular survival of *B. pertussis* inside MPI cells. Bars represent the mean of  
485 quadruplicates with the standard deviation (SD). (B) MPI cell viability assessed 24h after  
486 infection using MTT assay. Lines represent the mean of five replicates. Data are  
487 representatives of 3 independent experiments.

488 **Figure 8. Cytokine expression and secretion by *B. pertussis*-infected MPI cells.** (A)  
489 Expression of *tnf*, *il-1b*, *il-6* and *mip-2a* transcripts in MPI cells cultured in medium  
490 containing or not GM-CSF, 6h post-infection. (B) Cytokines production measured by ELISA  
491 in the supernatants of *B. pertussis*-infected cells 24h post-infection. The results represent the  
492 median of triplicates or quadruplicates. Statistical test used: Mann-Whitney. Data are  
493 representatives of 3 independent experiments. \*: p-value < 0,05.

494 **Figure 9. STAT3 activation in MPI cells infected with *B. pertussis*.** (A) Immunoblot of p-  
495 STAT3 (Tyr705), p-STAT3 (Ser727), STAT3 and  $\beta$ -actin of MPI cell lysate infected with *B.*  
496 *pertussis*. NI : Non-infected cells, BP : *B. pertussis* infected-cells. (B) Fold-protein expression  
497 of p-STAT3 (Tyr705), p-STAT3 (Ser727), STAT3, 4h post-infection. Results represent the  
498 median. Data are representatives of 3 independent experiments.

499 **Table 1. MPI cell cycle progression to G2 phase following *B. pertussis* infection.** Table  
500 showing the percentage of MPI cells in the different phases of the cell cycle analysed by FACS.  
501 The results represent the mean with standard deviation (SD).

502

## 503 **Supplementary material**

504 **Figure S1. Characterisation of MPI cell phenotype in FACS after GM-CSF starvation.**  
505 (A) Representative flow cytometry charts of SiglecF, CD11b, CD11c, CD32, F4/80, Ly6G  
506 expression by MPI cells. (B) Percentage of total MPI cells expressing F4/80, CD11b, CD11c,  
507 CD32 after GM-CSF starvation. The results represent the mean.

508 **Figure S2. Characterisation of MPIs cell cycle in FACS after GM-CSF starvation.** Cell  
509 cycle analysis of MPIs cells cultivated in the presence (A and B) or absence of GM-CSF (C  
510 and D) by FACS. A and C show three-dimensional flow cytometry of propidium iodide  
511 stained cells. B and D show the relative proportion of cells in each phase of the cycle and was  
512 calculated from A and C with the Dean-Jett-Fox model using FlowJo software. Experiment  
513 was repeated at least three times, and the data shown here are representative of the results  
514 obtained.

515

516 **Table T1. Primers for the genes of interest analyzed by qRT-PCR.** SP: Sense primer;  
517 ASP: Anti-Sense primer

518

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