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## **Indirect Toll-like receptor 5-mediated activation of conventional dendritic cells promotes the mucosal adjuvant activity of flagellin in the respiratory tract**

Delphine Fougeron, Laurye van Maele, Pascal Songhet, Delphine Cayet, David Hot, Nico van Rooijen, Hans-Joachim Mollenkopf, Wolf-Dietrich Hardt, Arndt G. Benecke, Jean-Claude Sirard

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1 **Indirect Toll-like Receptor 5-mediated activation of conventional dendritic**  
2 **cells promotes the mucosal adjuvant activity of flagellin in the respiratory**  
3 **tract<sup>1</sup>**

4  
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6 Hot<sup>\*,†,‡,§</sup>, Nico Van Rooijen<sup>||</sup>, Hans-Joachim Mollenkopf<sup>#</sup>, Wolf-Dietrich Hardt<sup>¶</sup>, Arndt G. Benecke<sup>\*\*</sup>,  
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14  
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25  
26 Running title: Nasal flagellin activates lung conventional dendritic cells

27 **Wordcount: 3000**

28 **Abstract**

29

30 The toll-like receptor 5 agonist (TLR5) flagellin is an effective adjuvant for vaccination. Recently, we  
31 demonstrated that the adaptive responses stimulated by intranasal administration of flagellin and  
32 antigen were linked to TLR5 signaling in the lung epithelium. The present study sought to identify the  
33 antigen presenting cells involved in this adjuvant activity. We first found that the lung dendritic cells  
34 captured antigen very efficiently in a process independent of TLR5. However, TLR5-mediated  
35 signaling specifically enhanced the maturation of lung dendritic cells. Afterward, the number of  
36 antigen-bound and activated conventional dendritic cells (both CD11b<sup>+</sup> and CD103<sup>+</sup>) increased in the  
37 mediastinal lymph nodes in contrast to monocyte-derived dendritic cells. These data suggested that  
38 flagellin-activated lung conventional dendritic cells migrate to the draining lymph nodes. The lymph  
39 node dendritic cells, in particular CD11b<sup>+</sup> cells, were essential for induction of CD4 T-cell response.  
40 Lastly, neutrophils and monocytes were recruited into the lungs by flagellin administration but did not  
41 contribute to the adjuvant activity. The functional activation of conventional dendritic cells was  
42 independent of direct TLR5 signaling, thereby supporting the contribution of maturation signals  
43 produced by flagellin-stimulated airway epithelium. In conclusion, our results demonstrated that  
44 indirect TLR5-dependent stimulation of airway conventional dendritic cells is essential to flagellin's  
45 mucosal adjuvant activity.

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47 **Wordcount: 205**

48

## 49 **Introduction**

50

51 Many pathogens of public health concern colonize the respiratory tract in general and the lung  
52 mucosa in particular. Mucosal vaccines induce a local adaptive immune response (i.e. secretory  
53 antibodies and mucosal T cells) and constitute a unique means of preventing these infections [1]. The  
54 few commercially available mucosal vaccines contain live attenuated pathogens and are effective.  
55 However, so far mucosal adjuvant candidates to be formulated with subunit vaccines have not  
56 demonstrated sufficient levels of potency and safety.

57 Most vaccines are delivered systemically and use systemic adjuvants. In recent years, it  
58 appears that these adjuvants mimic activation of immunity by live microorganisms and thereby  
59 enhance appropriate memory responses to infections [2, 3]. A common feature is the transient  
60 induction of an innate pro-inflammatory response [2, 4-7]. Thus, adjuvants like MF59 and alum  
61 trigger the infiltration of polymorphonuclear neutrophils (PMNs) and antigen-presenting cells (APCs,  
62 i.e. inflammatory monocytes and/or dendritic cells (DCs)) at the injection site [5, 8-11]. They  
63 specifically induce the functional maturation of APCs, thereby promoting functions related to antigen  
64 presentation.

65 Dendritic cells (DCs) are the most professional APCs that drive adjuvant activity [12]. In non-  
66 lymphoid tissues (such as lung) and at steady state, conventional DCs (cDCs) that originate from a  
67 pre-DC precursor serve as sentinels [13]. cDCs are characterized by the expression of CD11c, MHCII,  
68 CD24 and ZBTB46 and one can distinguish in the lungs between CD11b<sup>+</sup> and CD103<sup>+</sup> cDCs [13, 14].  
69 When activated by systemic adjuvants, tissue DCs migrate to draining lymph nodes and give rise to  
70 migratory DCs that trigger adaptive immunity [15-18]. The CD11b<sup>+</sup> cDCs are mainly associated with  
71 the trigger of Th2 responses, whereas CD103<sup>+</sup> cDCs rather promote Th1/Th17 responses and cross-  
72 presentation [17, 19-21]. Upon vaccination or infection, inflammatory monocytes that have entered  
73 tissues via CCR2 can also differentiate into DCs [10-12, 22]. These monocyte-derived DCs (moDCs)  
74 express not only CD11c and MHCII but also CD11b and the monocyte/macrophage marker CD64.  
75 However they lack CD24 [17, 23]. Depending on the adjuvant or the inflammatory injury, moDCs  
76 elicit or reactivate CD4 and CD8 T cell responses [8, 11, 24]. To develop safe and potent mucosal

77 vaccines, it is thus necessary to determine their impact on cDCs and moDCs recruitment and  
78 activation.

79 Toll-like receptors (TLRs) are essential for the activation of adaptive immunity by DCs and  
80 TLR agonists represent therefore potent adjuvant candidates for future vaccines. Flagellins are  
81 detected by TLR5, which in turn drives adjuvant activity via systemic and mucosal immunization  
82 routes [25]. When delivered by systemic route, flagellin directly stimulates the maturation of spleen  
83 DCs and thus activates T and B cell responses [26-28]. When flagellin is administered by the  
84 intranasal (i.n.) route, radioresistant cells of the lung epithelium expressing TLR5 control the innate  
85 immune responses [29, 30]. Recently, the antibody and CD4 T cell responses stimulated i.n. by  
86 flagellin as a mucosal adjuvant were linked to TLR5 signaling in the lung epithelium [30, 31]. Hence,  
87 this study aims at characterizing the APCs involved in flagellin's mucosal activity.

88

## 89 **Materials and methods**

90

91 **Mice and reagents.** Animals were maintained in a specific pathogen-free facility (#A59-350009,  
92 Institut Pasteur de Lille) and all experiments complied with national regulations and ethical guidelines.  
93 Six to 10-weeks-old mice (C57BL/6, C3H/HeN, *Tlr5*<sup>-/-</sup>, *Cd11c-DTR*, CD45.1, OT-II on the C57BL/6  
94 background) were obtained from Janvier, Charles River or bred in house. To generate bone marrow  
95 (BM) chimeric mice, wild type C57BL/6 (WT) or *Tlr5*<sup>-/-</sup> mice were lethally irradiated (11 Gray) and  
96 intravenously (i.v.) injected with 3-5 x10<sup>6</sup> BM cells. After 12-14 weeks, the reconstitution was  
97 assessed by flow cytometry using CD45.1 and CD45.2 markers. Flagellin (FliC) from *Salmonella*  
98 *enterica* Serovar Typhimurium, as well as “flagellin<sub>TLR5mut</sub>” (signaling motif [QRVRELAV] changed  
99 in the non signaling motif [DTVKVKAT]) were prepared as described [32]. Endograde ovalbumin  
100 (OVA) was purchased from Hyglos, AlexaFluor® 647 ovalbumin (OVA-AF647) from Molecular  
101 Probes and Hen Egg Lysosyme (HEL) from Appligene. All proteins were free of or depleted of  
102 endotoxin activity using Triton X-114 and/or Detoxi-Gel Affinity Columns (Pierce).

103

104 **Immunization and sampling.** Mice were anesthetized by intraperitoneal (i.p.) injection of ketamine-  
105 xylazine and immunized i.n. with 30µL of PBS containing OVA (10µg), OVA-AF647 (30µg) or HEL  
106 (800µg) ± flagellin (1µg). Mice were euthanized by i.p. injection of sodium pentobarbital and perfused  
107 lungs, MdLNs, cervical lymph nodes (CLN), spleen, or blood were collected. Intravenous injection of  
108 CD45-PE-Cy7 antibody was performed 5min prior to sampling to specifically label vasculature-  
109 associated or marginated cells [33]. To deplete DC, *Cd11c-DTR* → WT mice were i.p. injected with  
110 600ng and 200ng diphtheria toxin (DTX) 48h and 24h respectively before i.n. immunization as  
111 described [34].

112

113 **Flow cytometry.** Cells were isolated from tissues by digestion with collagenase IA (1mg/mL, Sigma)  
114 and DNase I (40µg/ml, Sigma) for 30 min at 37°C. The lung hematopoietic cells were recovered after  
115 centrifugation on Percoll 20%. After incubation with anti-CD16/CD32 Fc block (2.4G2), cells were  
116 stained with specific antibodies: CD45 (30F11), CD3ε (145-2C11), CD19 (1D3), CD4 (RM4-5), Vα2-

117 TCR (B20.1), CD11c (N418), CD24 (M1/69), CD64 (X54-5/7.1), MHCII (M5/114.5.2) specific for I-  
118 A<sup>b</sup>/I-E<sup>b</sup>/I-A<sup>k</sup>, CD103 (2E7), CD11b (M1/70), Ly6C (AL21), Ly6G (1A8), CCR2 (475301), CD86  
119 (GL-1), CD80 (16-10A1) (Becton Dickinson, BioLegend and eBioscience). C4H3-biotinylated  
120 antibody was used to detect HEL<sub>46-61</sub> peptide loaded on I-A<sup>k</sup> [26, 35]. Intracellular staining of IL-  
121 12 p40 (C15.6) was performed using cells incubated for 3h at 37°C with brefeldin A (1µg/mL, Sigma-  
122 Aldrich) and fixation/permeabilization kit from eBioscience. Data were collected on a BD LSR  
123 Fortessa and analyzed with BD FACSDiva or FlowJo. Cells were sorted on a BD FACSAria.

124

125 **Adoptive transfer of CD4 T cells.** CD4 lymphocytes were isolated from lymphoid organs of OT-II  
126 mice by negative selection (CD4 isolation kit, Miltenyi Biotec). Cells were stained with 5µM of  
127 carboxyfluorescein diacetate succinimidyl ester (CFSE) or 10µM of CellTrace™ Violet (CTV)  
128 (Invitrogen) and 5×10<sup>6</sup> cells were injected intravenously into animals. One day later, mice were  
129 immunized i.n. with PBS or OVA (20µg) ± flagellin. Tissues were sampled at day 5 for analysis of  
130 fluorescence dilution in T cells.

131

132 **Antigen presentation assays.** C57BL/6 mice were immunized i.n. with flagellin and OVA (47.5µg),  
133 MdLNs were sampled 18h later, and APCs were FACS sorted. APCs were cultured in round bottom  
134 96 wells plate with 3-5×10<sup>5</sup> CTV-stained OT-II cells at a ratio of 1:10. At day 5, cell proliferation was  
135 assessed by flow cytometry. The cytokines IL-13 and IFN-γ were detected in supernatants using  
136 ELISA kits (eBioscience).

137

138 **Statistical analysis.** Results are expressed as mean ± SEM. Mann-Whitney test was used to assess  
139 statistically significant between groups.

140

## 141 **Results**

142

### 143 **Flagellin promotes the recruitment of conventional dendritic cells into lung draining lymph** 144 **nodes**

145 To dissect flagellin's mode of action as an i.n. adjuvant, we investigated the early dynamic of  
146 lung DCs including cDCs and moDCs, as well as monocytes and PMNs (**Fig.1**). As previously  
147 described [29, 36, 37], our experiments revealed a recruitment of PMNs (Ly6C<sup>+</sup>Ly6G<sup>+</sup>) as early as 3h  
148 post-instillation that peaked at 6h (**Fig.1B**). Monocytes (Ly6G<sup>neg</sup>Ly6C<sup>+</sup>) were recruited by flagellin  
149 treatment in similar kinetics. These cells were also found to express CCR2 and CX3CR1, features of  
150 inflammatory monocytes (**data not shown**). In contrast, we observed a consistent but not significant  
151 decrease in the numbers of lung CD11b<sup>+</sup> or CD103<sup>+</sup> DCs at 6h (**Fig.1B**). Analysis of lung CD11b<sup>+</sup> DC  
152 revealed that cDC (CD24<sup>+</sup>) and moDC (CD64<sup>+</sup>) were similarly altered by flagellin immunization.  
153 Interestingly, the increase of lung monocyte counts was not associated with similar changes in  
154 moDCs, suggesting that monocytes did not give rise to moDC.

155 The MdLNs that drain the lung are known to be a major site for the induction of T cell-  
156 mediated immunity following flagellin administration [30, 31]. Relative to the lungs, significant  
157 changes in the MdLN counts of CD11b<sup>+</sup> and CD103<sup>+</sup> DCs were observed after i.n. administration of  
158 flagellin or flagellin plus antigen, relative to controls (**Fig.1C-D**). The changes in DC numbers were  
159 observed after 18h treatment (but not after 6h) and rapidly returned to baseline. We further established  
160 that the increase of MdLN CD11b<sup>+</sup> DCs was due to cDCs but not moDCs, given their expression of  
161 CD24 and the lack of CD64 expression (**Fig.1E**). Importantly, DC recruitment was not detected in the  
162 CLNs draining the nasal cavity (**Fig.1F**). In summary, intranasal administration of flagellin triggers an  
163 early recruitment of PMN and monocytes into the lung and an increase of cDC numbers in MdLNs.

164

### 165 **Antigen-bound dendritic cells predominates in the airways and draining lymph nodes**

166 In order to track the cells that had captured the antigen, fluorescent ovalbumin (OVA-AF647)  
167 was administered intranasally (**Fig.2**). As early as 3h post-immunization, lung cells were associated  
168 with the fluorescent antigen (**data not shown**). Six hours post-administration of OVA-AF647, we  
169 found that 50% of CD11b<sup>+</sup> DCs, 70% of CD103<sup>+</sup> DCs, 20% of PMNs and 10% of monocytes were



170 bound to antigen (**Fig.2A**). Remarkably, the same proportion of antigen-bound cells was found 6h  
171 after administration of flagellin plus OVA-AF647. However, the number of monocytes and PMNs  
172 bound to OVA increased >3-fold and >20-fold, respectively, thereby correlating with the magnitude of  
173 these cells' infiltration (**Fig.2B**). In contrast, the CD11b<sup>+</sup> and CD103<sup>+</sup> DC counts bound to OVA did  
174 not change significantly after flagellin treatment. We also investigated the localization of OVA-bound  
175 lung cells by injecting an intravenous pulse of CD45-specific antibody (**Fig.2C-D**). Interestingly,  
176 antigen-bearing monocytes were mostly marginated (i.e. in the vasculature) whereas DCs were mostly  
177 interstitial. In contrast, PMNs shifted from marginated to interstitial position after flagellin  
178 stimulation, revealing the translocation of PMN into the bronchoalveolar compartment. Taken as a  
179 whole, these data suggest that lung DCs have a key role in antigen capture following intranasal  
180 vaccination.

181 We next investigated whether cells that had captured fluorescent OVA can be found in the  
182 MdLNs. In contrast to lungs, we did not detect any significant changes of DC harboring antigen in the  
183 MdLN 6h post-administration of flagellin (**data not shown**). However, the number of OVA-bound  
184 CD11b<sup>+</sup> and CD103<sup>+</sup> MdLN DCs was significantly enhanced at 18h (**Fig.3A**). We did not detect any  
185 antigen-bound PMN or monocytes in MdLN. Moreover, there was not any flagellin-dependent  
186 increase of antigen-bound cells in the CLNs draining the nasal cavity. These observations suggest that  
187 antigen capture by lung DC is followed by migration of cDC into MdLN.

188 Finally, we assessed the potential contribution of infiltrating monocytes or PMNs to flagellin's  
189 adjuvant activity by performing experiments with clodronate liposomes, *Ccr2*<sup>-/-</sup> mice and Ly6G-  
190 specific depleting antibodies (**Supplementary Fig.1**). Animals depleted or deficient in PMNs or  
191 monocytes responded in much the same way as control animals did to i.n. immunization - suggesting  
192 that monocytes and PMNs do not modulate the adjuvant activity.

193

#### 194 **Dendritic cells are functionally activated by flagellin**

195 Since lung DCs from control and flagellin-immunized animals captured similarly antigen, we  
196 next assessed whether flagellin impact on DC activation Microarray analysis of transcriptional  
197 changes over time in lung highlighted that the DC maturation pathway and the functions related to  
198 antigen presentation were upregulated (**Supplementary Fig.2**). In contrast, these patterns were not

199 observed in the blood. We next observed that both CD11b<sup>+</sup> and CD103<sup>+</sup> lung cDCs upregulated the  
200 expression of MHCII and the co-stimulatory molecules CD80 and CD86 as soon as 6h post-  
201 immunization (**Supplementary Fig.3A-C**). In addition, the lung cDCs upregulated functions of  
202 processing and presentation of antigen since immunization with hen egg lysozyme (HEL) and flagellin  
203 significantly increased staining with the C4H3 antibody that recognizes the MHCII-loaded HEL<sub>46-61</sub>  
204 (**Supplementary Fig.3D**).

205 The flagellin administration was also associated with functionally activated cDCs in MdLNs  
206 18h post-immunization as evaluated by the upregulation of CD80, CD86 as well as C4H3 staining  
207 (**Fig.3B-D**). Lastly, MdLN CD11b<sup>+</sup> but not CD103<sup>+</sup> cDCs were shown to produce the T-cell activating  
208 cytokine IL-12 p40 (**Fig.3E-F**). Altogether, these results suggest that flagellin signaling enhances the  
209 functional activation of lung cDCs and their migration to the MdLNs.

210

### 211 **CD11b<sup>+</sup> cDCs are crucial for the flagellin-mediated CD4<sup>+</sup> T cell activation**

212 To ascertain the role of cDCs, we performed experiments with chimera mice harboring  
213 *Cd11c-DTR* bone marrow that can be injected with DTX to eliminate lung DCs (**Fig.4A**). Passively  
214 transferred, OVA-specific CD4<sup>+</sup> OT-II cells were monitored as a guide to adjuvant activity. Flagellin  
215 and OVA strongly stimulated the proliferation of OT-II cells both in MdLNs from control wild type  
216 animals and control *Cd11c-DTR* chimera animals (**Fig.4B-C**). DTX treatment of *Cd11c-DTR* mice  
217 prior to immunization abrogated the proliferation of the OT-II cells. Interestingly, DC depletion did  
218 not impact on the flagellin-mediated lung innate response, as assessed by production of the chemokine  
219 CCL20 (**Fig.4D**). Therefore, these data strongly suggest that mucosal cDCs are essential for the  
220 adjuvant activity of i.n. flagellin.

221 The cDCs' ability to present antigen was further analyzed *in vitro*. OT-II lymphocytes were  
222 cultured with total DCs, CD11b<sup>+</sup> or CD103<sup>+</sup> DCs that were sorted from the MdLNs of animals  
223 immunized i.n. with flagellin and OVA. We found that CD11b<sup>+</sup> DCs efficiently activated OT-II cells  
224 and thus prompted them to proliferate and secrete the T cell-specific cytokines IFN $\gamma$  and IL-13. In  
225 these conditions, CD103<sup>+</sup> DCs hardly stimulated proliferation or cytokine production (**Fig.4E-G**).  
226 Similar results were obtained by injecting sorted MdLN DCs into mice adoptively transferred with

227 OT-II cells (**Supplementary Fig.4**). These data demonstrated that CD11b<sup>+</sup> MdLN cDCs are essential  
228 components in the flagellin's adjuvant activity by stimulating CD4<sup>+</sup> T cell responses.

229

### 230 **Direct TLR5 signaling in DCs is not required for the cells' functional activation**

231 Intranasal adjuvant activity of flagellin depends on TLR5 and is abrogated in *Tlr5*<sup>-/-</sup> mice or  
232 mice immunized with flagellin mutated within the TLR5 motif, i.e. flagellin<sub>TLR5mut</sub> [30, 32]. We  
233 therefore studied whether flagellin-mediated MdLN DC activation depends on TLR5 signaling.  
234 Notably, we did not detect any activation of MdLN cDCs in *Tlr5*<sup>-/-</sup> mice in contrast to the situation in  
235 WT animals (**Fig.5A-B**). Similarly, intranasal immunization of WT animals with flagellin<sub>TLR5mut</sub>, did  
236 not induce any activation of MdLN DCs in contrast to native flagellin (**Fig.5C**).

237 To determine whether nasal administration of flagellin directly activate airway DCs, we  
238 generated chimeric mice by transferring a combination of *Tlr5*<sup>-/-</sup> and WT bone marrow (**Fig.5D**).  
239 Activation was measured by the expression of co-stimulatory molecules in CD45.1-expressing DC  
240 (i.e. WT) and CD45.2-expressing DC (i.e. *Tlr5*<sup>-/-</sup>). As previously reported, intraperitoneal  
241 administration of flagellin only induced activation of WT spleen DC but not *Tlr5*<sup>-/-</sup> DC (**Fig.5E**). This  
242 systemic DC activation was therefore a process dependent on direct TLR5 signaling. In contrast, after  
243 intranasal flagellin administration, MdLN DC activation occurred independently of TLR5 expression  
244 on DCs (**Fig.5F-G**), indicating that *Tlr5*<sup>-/-</sup> MdLN DC are transactivated by WT cells. Using chimera  
245 where *Tlr5*<sup>-/-</sup> animals were transferred with WT cells and the opposite condition, we found that MdLN  
246 DC activation required *Tlr5* expression in the radioresistant cell compartment (**Supplementary Fig.5**).  
247 Interestingly, *Tlr5* mRNA levels were respectively 8-fold lower in lung DC compared to airway  
248 epithelial cells (**Supplementary Fig.5**). In summary, these data suggest that flagellin's effect on cDC  
249 activation was independent of direct TLR5 signaling in DCs but requires instruction by TLR5-  
250 dependent mechanisms in epithelial cells.

251

252 **Discussion**

253

254         The development of novel prophylactic strategies requires knowledge of the APCs involved in  
255 vaccine efficacy. Our present results demonstrate that the intranasal adjuvant activity of flagellin is  
256 linked to the functional activation of lung DCs and MdLN cDCs. Our data suggest that flagellin  
257 enhances the ability of lung cDC to present antigen and to migrate to draining lymph nodes for CD4<sup>+</sup>  
258 lymphocyte stimulation. Furthermore, cDC activation does not involve direct TLR5 signaling but is  
259 likely dependent on transactivation signals originating from TLR5-expressing airway epithelial cells.  
260 Finally, we provided strong evidence that transactivation does not rely on the expression of IL-25,  
261 TSLP, IL-33, IL-1 $\alpha$ , IL-1 $\beta$ , or IL-36 $\gamma$ .

262         How DCs are activated is an important parameter in the development of adjuvants. A  
263 prominent concept is the direct activation of DCs by adjuvants [38]. The mucosal activity of flagellin  
264 requires epithelial TLR5 signaling in airways [29, 30]. The present data indicate that DCs are not the  
265 target cells of flagellin since they are activated by indirect TLR5 rather than direct TLR5 signaling,  
266 reinforcing the contribution of airway epithelium as flagellin target. This contrasts with the systemic  
267 route of flagellin immunization that directly stimulate TLR5 signaling in DCs (**Fig.5 and**  
268 **Supplementary Fig.5**) [27, 39]. Airway epithelial cells were also found essential to induce the DC  
269 activation in response to respiratory delivery of TLR4-dependent allergen that leads to asthma [40].  
270 Epithelial cytokines that activate lung DCs in response to mucosal challenge have been identified [41-  
271 44]. Defining the epithelial factors involved in the adjuvant effect of flagellin will open up  
272 unprecedented avenues in the vaccine field.

273         Our results demonstrated that lung DCs are activated and that MdLN cDCs, particularly  
274 CD11b<sup>+</sup> cDCs present antigen to CD4<sup>+</sup> T cells. The counts of MdLN cDCs rose during immunization  
275 and thus supported the migration of lung cDCs to lymph nodes. Lung cDCs efficiently capture antigen  
276 in absence of any stimulation since they are ideally positioned for antigen sampling [45]. Our data  
277 demonstrate that the main activity of flagellin as an adjuvant is to functionally activate DCs.  
278 Consistently, CD11b<sup>+</sup> DC have a key role in the mucosal adjuvant activity of cholera toxin [46].

279         The lung transcriptional signature induced by intranasal administration of flagellin shares  
280 features of various adjuvants [5-7, 21]. Adjuvants generally upregulate the pathways of acute phase

281 response and chemokine signaling, highlighting the recruitment of inflammatory cells. PMNs are  
282 mobilized in response to flagellin and the process is linked to the production of chemokines by the  
283 epithelial TLR5 signaling pathway [29, 36]. Our data suggest that PMNs do not regulate the flagellin's  
284 adjuvant activity (as for the adjuvant MF59 [47]). Flagellin administration also promoted the  
285 recruitment of monocytes into lung but monocytes and moDCs are not required for flagellin-mediated  
286 activity. It is noteworthy that in asthma, high doses of allergen stimulates lung moDC migration to the  
287 lymph nodes [20]. In our setting, the low antigen doses and the transient innate response stimulated by  
288 flagellin may impair differentiation into moDCs. The observation that flagellin's adjuvant activity is  
289 dissociated from inflammatory cell recruitment is a fascinating perspective for vaccine improvement.

290         The development of mucosal adjuvants requires a detailed understanding of the transactivating  
291 factors and the signals that bypass the tolerogenic imprinting of the mucosa on DCs. In conclusion,  
292 this study opens up new avenues for designing safer adjuvants with a less intense pro-inflammatory  
293 response and fewer side effects.

294

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296

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300 France for flow cytometry and cell sorting, respectively.

301

302 **Authorship**

303 Contribution: D.F. planned studies, performed experiments, analyzed data, and wrote the manuscript;  
304 L.V.M. performed experiments and analyzed data. D.C. and P.S. performed experiments. N.V.R., and  
305 W.D.H. contributed vital reagents. DH, H.J.M. and A.G.B. performed experiments and analyzed  
306 microarray data. J.C.S. supervised the project and wrote the manuscript.

307

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309

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313

314 **Figure legends**

315

316 **Figure 1: Analysis of recruitment of neutrophils, monocytes and dendritic cells into the lungs**  
317 **and draining lymph nodes following flagellin immunization.** C57BL/6 mice (n=4) were treated i.n.  
318 with either flagellin, ovalbumin (OVA), or flagellin plus OVA. Lung, mediastinal (MdLN) or cervical  
319 (CLN) lymph nodes samples were collected at the indicated time points for flow cytometry analysis.  
320 (A) Gating strategy for the identification of lung cells. After gating on CD45<sup>+</sup>, lung cells were  
321 identified as follows: cDCs (lin<sup>neg</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD24<sup>+</sup>CD11b<sup>+/neg</sup>CD103<sup>+/neg</sup>), moDCs  
322 (lin<sup>neg</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>), monocytes (CD11b<sup>high</sup>Ly6C<sup>high</sup>Ly6G<sup>neg</sup>) and PMNs  
323 (CD11b<sup>high</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup>). The same strategy was used for MdLN and CLN cells. (B,C) Absolute  
324 counts of lung total PMNs, monocytes, CD11b<sup>+</sup> cDCs, moDCs, and CD103<sup>+</sup> cDCs over time in lungs  
325 (B) and MdLNs following flagellin immunization (C). (D) Numbers of CD11b<sup>+</sup> and CD103<sup>+</sup> DC in  
326 MdLNs 18h post-immunization with OVA or flagellin plus OVA. (E) Analysis of composition of  
327 CD11b<sup>+</sup> DC in MdLNs. (F) Numbers of CD11b<sup>+</sup> and CD103<sup>+</sup> DC in CLNs 18h post-immunization  
328 with OVA or flagellin plus OVA. Results are expressed as the mean ± SEM and are representative of  
329 2-5 experiments (\* *p* <0.05).

330

331 **Figure 2: Analysis of antigen uptake by lung neutrophils, monocytes and dendritic cells.**  
332 C57BL/6 mice (n=4) were treated i.n. with fluorescent ovalbumin (OVA-AF647) in the presence or  
333 absence of flagellin. Lung samples were collected at 6h for flow cytometry analysis. (A) Absolute  
334 counts of OVA-positive PMNs (CD45<sup>+</sup>CD11b<sup>high</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup>), monocytes  
335 (CD45<sup>+</sup>CD11b<sup>high</sup>Ly6C<sup>high</sup>Ly6G<sup>neg</sup>), CD11b<sup>+</sup> and CD103<sup>+</sup> DCs  
336 (lin<sup>neg</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+/neg</sup>CD103<sup>+/neg</sup>). (B) Representative histograms of antigen uptake  
337 by cell type. For each cell type, the number indicates the percentage of OVA-positive cells among the  
338 total population considered. (C-D) Six hours post-immunization, mice were injected i.v. with anti-  
339 CD45-PeCy7 (1µg) and lung tissues were sampled 5 minutes later. Cells stained by anti-CD45-PeCy7  
340 are defined as marginated cells. (C) Percentage of margination was determined for monocytes, DC,  
341 and PMN. (D) Representative plots showing the correlation between antigen uptake and cell



342 localization. Results are expressed as the mean  $\pm$  SEM, and are representative of 2-4 experiments (\* p  
343 <0.05).

344

345 **Figure 3: Draining lymph nodes CD11b<sup>+</sup> cDC process antigens and exhibit functional**  
346 **maturation after flagellin intranasal administration.** Mice (n=3-4) were immunized i.n. and  
347 mediastinal lymph nodes (MdLN) were sampled at 18h for flow cytometry analysis. Dendritic cells  
348 were defined as lin<sup>neg</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+/-</sup>CD103<sup>+/-</sup>. (A-C) C57BL/6 animals were  
349 immunized i.n. with fluorescent ovalbumin (OVA-AF647) or flagellin and OVA-AF647. (A) Absolute  
350 numbers of antigen-positive MdLN DCs. (B) Representative histograms of CD80 and CD86  
351 expression by MdLN DCs. (C) Quantitative analysis of CD86 expression in DCs. (D) C3H/HeN  
352 animals were immunized i.n. with either hen egg lysozyme (HEL), flagellin, or flagellin and HEL.  
353 Antigen presentation by DCs was assessed with C4H3 antibody that detects HEL peptide loaded on I-  
354 A<sup>k</sup>. Fluorescence of the C4H3 antibody was normalized against the I-A<sup>k</sup> fluorescence. (E-F) C57BL/6  
355 were immunized i.n. with PBS or flagellin. (E) Representative histograms of IL-12 p40-producing  
356 DCs. (F) Percentage of IL-12 p40-producing CD11b<sup>+</sup> and CD103<sup>+</sup> DC among the total population of  
357 DC. Results are expressed as the mean  $\pm$  SEM and are representative of 2-4 experiments (\* p <0.05).

358

359 **Figure 4: CD11b<sup>+</sup> dendritic cells are essential for flagellin's mucosal adjuvant activity.**

360 (A-D) C57BL/6 (WT) and *Cd11c*-DTR  $\rightarrow$  WT chimera mice (n=3-4) were treated twice i.p. with PBS  
361 or diphtheria toxin (DTX) (200 ng 24h before immunization and 600 ng at immunization). (A)  
362 Representative plots of DC depletion in the spleen and lung. Using flow cytometry, dendritic cells  
363 were defined as lin<sup>neg</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+/-</sup>CD103<sup>+/-</sup>. Numbers indicate the DC as  
364 percentage of CD45<sup>+</sup> cells 24h after DTX injection. (B-C) WT and *Cd11c*-DTR  $\rightarrow$  WT mice were  
365 adoptively transferred with CFSE-labeled OT-II CD4<sup>+</sup> T cells that are specific for ovalbumin (OVA)  
366 and treated i.p. with DTX or PBS. After 24h, Animals were immunized i.n. with PBS or flagellin and  
367 OVA and treated i.p. with DTX or PBS. The proliferation of OT-II cell was analyzed 5 days after  
368 immunization in the spleen and the mediastinal lymph nodes (MdLN). Abrogation of T cell response  
369 by DC depletion is shown as representative plots (B) and quantification of division (C). Plots show the  
370 CFSE fluorescence of CD4<sup>+</sup>V $\alpha$ 2<sup>+</sup> OT-II cells and are representative of two experiments. (D) Lung

371 innate response to flagellin after DC depletion. WT and *Cd11c*-DTR → WT mice were DTX-treated  
372 and immunized i.n. with PBS or flagellin and OVA. CCL20 production was measured in the serum 6h  
373 after the i.n. administration as a marker of lung activation. (E-G) C57BL/6 mice (n=16) were  
374 immunized i.n. with flagellin and OVA and the MdLN were sampled 18h later. DCs were sorted as the  
375 total DC population ( $\text{lin}^{\text{neg}}\text{CD45}^+\text{CD11c}^+\text{MHCII}^+$ ),  $\text{CD11b}^+$  and  $\text{CD103}^+$  DC subsets. DCs ( $5 \times 10^4$ )  
376 were cultured with CTV-labeled OT-II cells ( $5 \times 10^5$ ) for 5 days. (E-F) T cell proliferation induced by  
377 DCs is shown as representative histograms of CTV dilution (E) and quantification of division as a  
378 percentage of total OT-II cells (F). (G) Analysis of cytokine production in the DC-OT-II co-culture  
379 supernatants. The results are representative of three experiments (\*  $p < 0.05$ ).

380

381 **Figure 5: Dendritic cell maturation following intranasal administration of flagellin does not**  
382 **require direct activation through TLR5 signaling.**

383 (A-B) *Tlr5*<sup>-/-</sup> or C57BL/6 (WT) mice (n=3-4) were immunized i.n. with PBS or flagellin. (C) C57BL/6  
384 mice (n=3-4) were immunized i.n. with PBS, flagellin or flagellin mutated for the TLR5 signaling  
385 motif (flagellin<sub>TLR5mut</sub>). Cells isolated 18h after immunization from mediastinal lymph nodes (MdLN)  
386 were analyzed by flow cytometry. Dendritic cells (DC) were defined as  
387  $\text{lin}^{\text{neg}}\text{CD45}^+\text{CD11c}^+\text{MHCII}^+\text{CD11b}^{+/-}\text{CD103}^{+/-}$ . (A-C) Quantitative analysis of CD80 and CD86  
388 expression. (D) Experimental strategy of mixed bone marrow chimera. Bone-marrow from *Tlr5*<sup>-/-</sup>  
389 [CD45.2] and WT [CD45.1] (1:1 ratio) animals were injected into lethally irradiated WT [CD45.2]  
390 mice. Three months after engraftment (>95% reconstitution of DC compartment), chimera mice (n=3-  
391 5) were immunized with flagellin by intravenous (E) or intranasal route (F-G). The functional  
392 maturation of spleen DC (E) or MdLN DC (F-G) was assessed 18h after PBS or flagellin  
393 administration as described in (A-C). (E) Quantitative analysis of CD86 expression in spleen *Tlr5*<sup>-/-</sup>  
394 and WT DC. (F) Representative plots of CD86 expression by *Tlr5*<sup>-/-</sup> and WT MdLN DC in response to  
395 intranasal injection of PBS or flagellin. (G) Mean CD86 expression in *Tlr5*<sup>-/-</sup> and WT MdLN DC. Data  
396 represent the mean ± SEM and are representative of 2-4 experiments (\* $p < 0.05$ ).

397

398 **References**

399

400 [1] Lycke N. Recent progress in mucosal vaccine development: potential and limitations. *Nat Rev*  
401 *Immunol.* 2012;12:592-605.

402 [2] Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, et al. Synthetic  
403 double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. *J*  
404 *Exp Med.* 2011;208:2357-66.

405 [3] Blander JM, Sander LE. Beyond pattern recognition: five immune checkpoints for scaling the  
406 microbial threat. *Nat Rev Immunol.* 2012;12:215-25.

407 [4] Buonaguro L, Pulendran B. Immunogenomics and systems biology of vaccines. *Immunol Rev.*  
408 2011;239:197-208.

409 [5] Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an  
410 aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune  
411 response leading to enhanced adaptive immunity. *J Immunol.* 2009;183:6186-97.

412 [6] Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, et al. Molecular and cellular  
413 signatures of human vaccine adjuvants. *Proc Natl Acad Sci U S A.* 2008;105:10501-6.

414 [7] Tritto E, Muzzi A, Pesce I, Monaci E, Nuti S, Galli G, et al. The acquired immune response to the  
415 mucosal adjuvant LTK63 imprints the mouse lung with a protective signature. *J Immunol.*  
416 2007;179:5346-57.

417 [8] Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant  
418 boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp*  
419 *Med.* 2008;205:869-82.

420 [9] Didierlaurent AM, Collignon C, Bourguignon P, Wouters S, Fierens K, Fochesato M, et al.  
421 Enhancement of Adaptive Immunity by the Human Vaccine Adjuvant AS01 Depends on Activated  
422 Dendritic Cells. *J Immunol.* 2014;193:1920-30.

423 [10] Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminum hydroxide and  
424 MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation  
425 toward dendritic cells. *J Immunol.* 2008;180:5402-12.

- 426 [11] Le Borgne M, Etchart N, Goubier A, Lira SA, Sirard JC, van Rooijen N, et al. Dendritic cells  
427 rapidly recruited into epithelial tissues via CCR6/CCL20 are responsible for CD8<sup>+</sup> T cell crosspriming  
428 in vivo. *Immunity*. 2006;24:191-201.
- 429 [12] Randolph GJ, Jakubzick C, Qu C. Antigen presentation by monocytes and monocyte-derived  
430 cells. *Curr Opin Immunol*. 2008;20:52-60.
- 431 [13] Meredith MM, Liu K, Darrasse-Jeze G, Kamphorst AO, Schreiber HA, Guermonprez P, et al.  
432 Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic  
433 cell lineage. *J Exp Med*. 2012;209:1153-65.
- 434 [14] Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, et al. Deciphering the  
435 transcriptional network of the dendritic cell lineage. *Nat Immunol*. 2012;13:888-99.
- 436 [15] Anandasabapathy N, Feder R, Mollah S, Tse SW, Longhi MP, Mehandru S, et al. Classical Flt3L-  
437 dependent dendritic cells control immunity to protein vaccine. *J Exp Med*. 2014.
- 438 [16] Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization  
439 through signaling specificity and transcriptional programming. *The EMBO journal*. 2014;33:1104-16.
- 440 [17] Langlet C, Tamoutounour S, Henri S, Luche H, Ardouin L, Gregoire C, et al. CD64 expression  
441 distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during  
442 intramuscular immunization. *J Immunol*. 2012;188:1751-60.
- 443 [18] Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-  
444 dependent CD11b<sup>+</sup> dendritic cells in human and mouse control mucosal IL-17 cytokine responses.  
445 *Immunity*. 2013;38:970-83.
- 446 [19] Furuhashi K, Suda T, Hasegawa H, Suzuki Y, Hashimoto D, Enomoto N, et al. Mouse lung  
447 CD103<sup>+</sup> and CD11b<sup>high</sup> dendritic cells preferentially induce distinct CD4<sup>+</sup> T-cell responses. *Am J*  
448 *Respir Cell Mol Biol*. 2012;46:165-72.
- 449 [20] Plantinga M, Guilliams M, Vanheerswynghels M, Deswarte K, Branco-Madeira F, Toussaint W,  
450 et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2  
451 cell-mediated immunity to house dust mite allergen. *Immunity*. 2013;38:322-35.
- 452 [21] Pesce I, Monaci E, Muzzi A, Tritto E, Tavarini S, Nuti S, et al. Intranasal administration of CpG  
453 induces a rapid and transient cytokine response followed by dendritic and natural killer cell activation  
454 and recruitment in the mouse lung. *J Innate Immun*. 2010;2:144-59.

455 [22] Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol.*  
456 2011;11:762-74.

457 [23] Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 Transcription Factor-  
458 Dependent CD11b(+) Dendritic Cells in Human and Mouse Control Mucosal IL-17 Cytokine  
459 Responses. *Immunity.* 2013;38:970-83.

460 [24] Iijima N, Mattei LM, Iwasaki A. Recruited inflammatory monocytes stimulate antiviral Th1  
461 immunity in infected tissue. *Proc Natl Acad Sci U S A.* 2011;108:284-9.

462 [25] Mizel SB, Bates JT. Flagellin as an adjuvant: cellular mechanisms and potential. *J Immunol.*  
463 2010;185:5677-82.

464 [26] Didierlaurent A, Ferrero I, Otten LA, Dubois B, Reinhardt M, Carlsen H, et al. Flagellin promotes  
465 myeloid differentiation factor 88-dependent development of Th2-type response. *J Immunol.*  
466 2004;172:6922-30.

467 [27] Kinnebrew MA, Buffie CG, Diehl GE, Zenewicz LA, Leiner I, Hohl TM, et al. Interleukin 23  
468 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances  
469 mucosal innate immune defense. *Immunity.* 2012;36:276-87.

470 [28] Uematsu S, Jang MH, Chevrier N, Guo Z, Kumagai Y, Yamamoto M, et al. Detection of  
471 pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. *Nat*  
472 *Immunol.* 2006;7:868-74.

473 [29] Janot L, Sirard JC, Secher T, Noulin N, Fick L, Akira S, et al. Radioresistant cells expressing  
474 TLR5 control the respiratory epithelium's innate immune responses to flagellin. *Eur J Immunol.*  
475 2009;39:1587-96.

476 [30] Van Maele L, Fougerson D, Janot L, Didierlaurent A, Cayet D, Tabareau J, et al. Airway structural  
477 cells regulate TLR5-mediated mucosal adjuvant activity. *Mucosal Immunol.* 2014;7:489-500.

478 [31] Wilson RH, Maruoka S, Whitehead GS, Foley JF, Flake GP, Sever ML, et al. The Toll-like  
479 receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nat*  
480 *Med.* 2012;18:1705-10.

481 [32] Nempont C, Cayet D, Rumbo M, Bompard C, Villeret V, Sirard JC. Deletion of flagellin's  
482 hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-  
483 dependent immunity. *J Immunol.* 2008;181:2036-43.

484 [33] Barletta KE, Cagnina RE, Wallace KL, Ramos SI, Mehrad B, Linden J. Leukocyte compartments  
485 in the mouse lung: distinguishing between marginated, interstitial, and alveolar cells in response to  
486 injury. *J Immunol Methods*. 2012;375:100-10.

487 [34] Van Maele L, Carnoy C, Cayet D, Songhet P, Dumoutier L, Ferrero I, et al. TLR5 signaling  
488 stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and  
489 mucosa. *J Immunol*. 2010;185:1177-85.

490 [35] Inaba K, Turley S, Iyoda T, Yamaide F, Shimoyama S, Reis e Sousa C, et al. The formation of  
491 immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of  
492 dendritic cells is regulated by inflammatory stimuli. *J Exp Med*. 2000;191:927-36.

493 [36] Honko AN, Mizel SB. Mucosal administration of flagellin induces innate immunity in the mouse  
494 lung. *Infect Immun*. 2004;72:6676-9.

495 [37] Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration  
496 of flagellin protects mice from *Streptococcus pneumoniae* lung infection. *Infect Immun*.  
497 2010;78:4226-33.

498 [38] Sporri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation  
499 and promote expansion of CD4+ T cell populations lacking helper function. *Nat Immunol*.  
500 2005;6:163-70.

501 [39] Flores-Langarica A, Marshall JL, Hitchcock J, Cook C, Jobanputra J, Bobat S, et al. Systemic  
502 flagellin immunization stimulates mucosal CD103+ dendritic cells and drives Foxp3+ regulatory T  
503 cell and IgA responses in the mesenteric lymph node. *J Immunol*. 2012;189:5745-54.

504 [40] Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite  
505 allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med*.  
506 2009;15:410-6.

507 [41] Iwasaki A. Mucosal dendritic cells. *Annu Rev Immunol*. 2007;25:381-418.

508 [42] Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from  
509 protection to immunopathology. *Annu Rev Immunol*. 2012;30:243-70.

510 [43] Pang IK, Ichinohe T, Iwasaki A. IL-1R signaling in dendritic cells replaces pattern-recognition  
511 receptors in promoting CD8(+) T cell responses to influenza A virus. *Nat Immunol*. 2013;14:246-53.

512 [44] Tortola L, Rosenwald E, Abel B, Blumberg H, Schafer M, Coyle AJ, et al. Psoriasiform  
513 dermatitis is driven by IL-36-mediated DC-keratinocyte crosstalk. *J Clin Invest.* 2012;122:3965-76.

514 [45] Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL. Regulation of immunological homeostasis  
515 in the respiratory tract. *Nat Rev Immunol.* 2008;8:142-52.

516 [46] Fahlen-Yrliid L, Gustafsson T, Westlund J, Holmberg A, Strombeck A, Blomquist M, et al.  
517 CD11c(high) dendritic cells are essential for activation of CD4+ T cells and generation of specific  
518 antibodies following mucosal immunization. *J Immunol.* 2009;183:5032-41.

519 [47] Calabro S, Tortoli M, Baudner BC, Pacitto A, Cortese M, O'Hagan DT, et al. Vaccine adjuvants  
520 alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen  
521 transport to draining lymph nodes. *Vaccine.* 2011;29:1812-23.

522

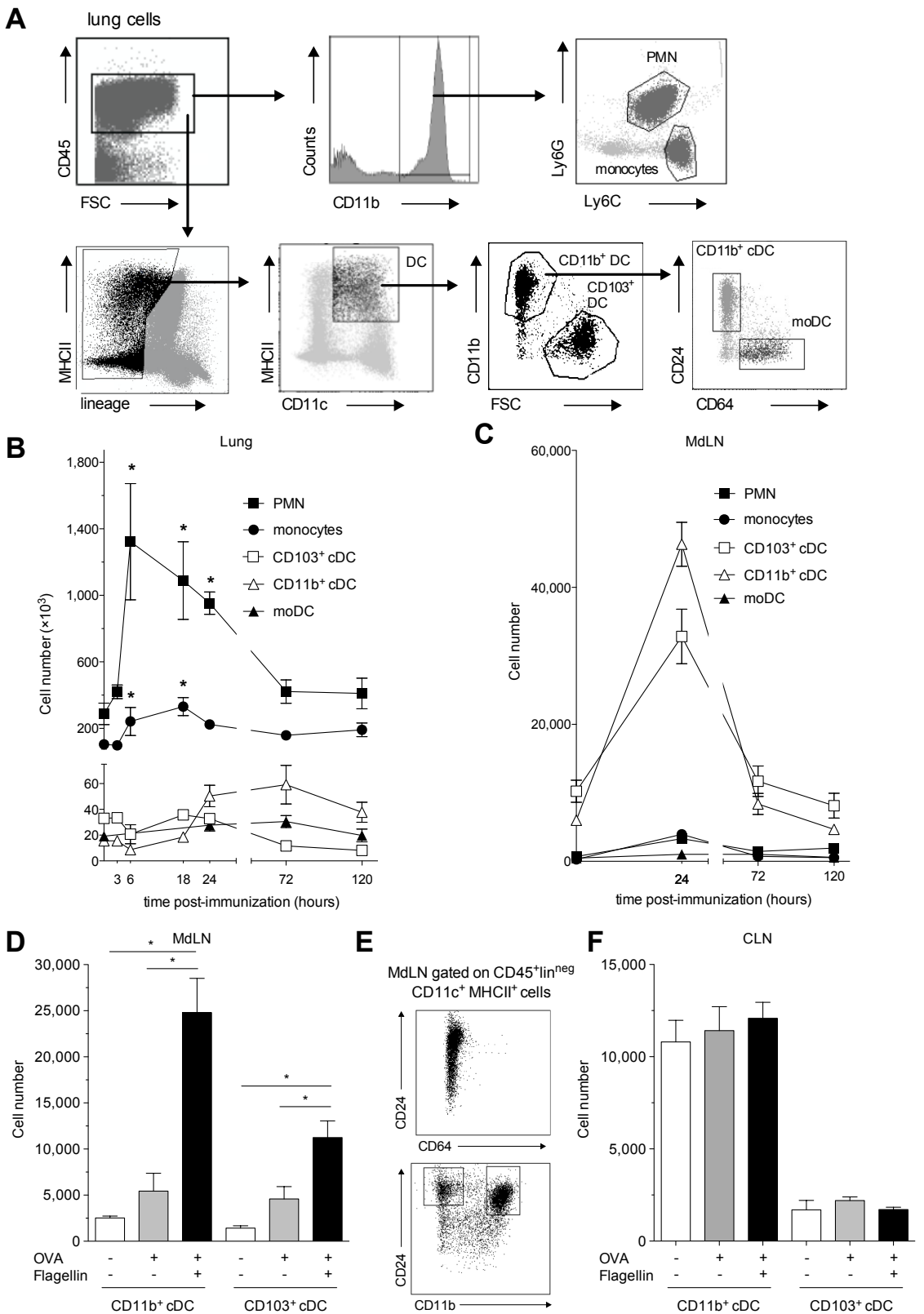


Figure 1: Fougeron *et al.*



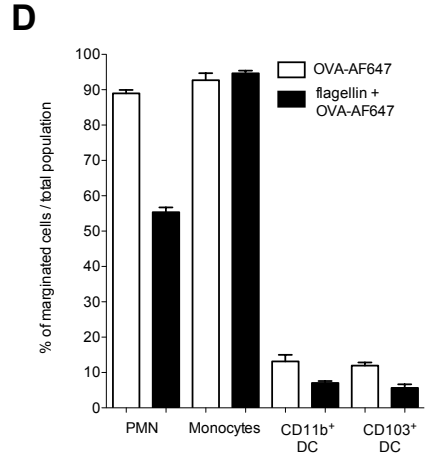
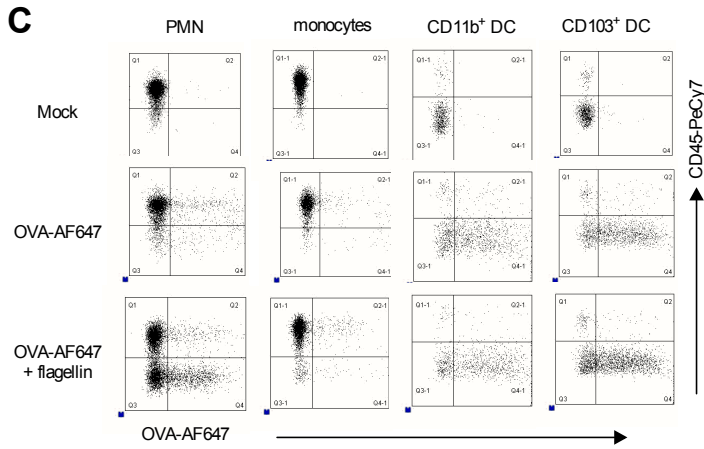
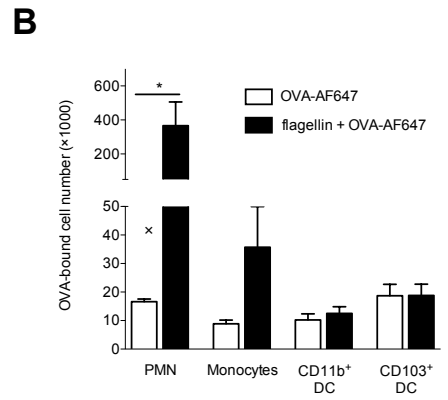
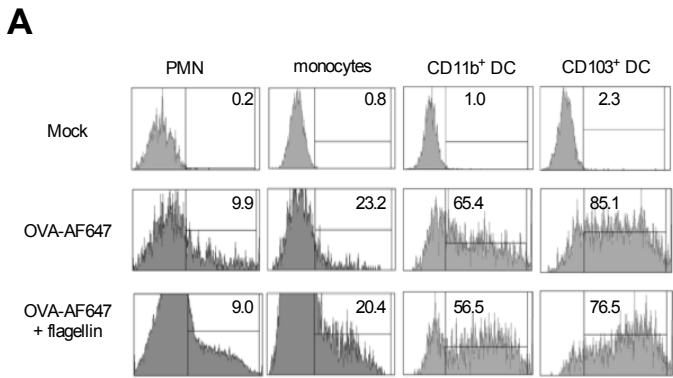


Figure 2: Fougeron *et al.*

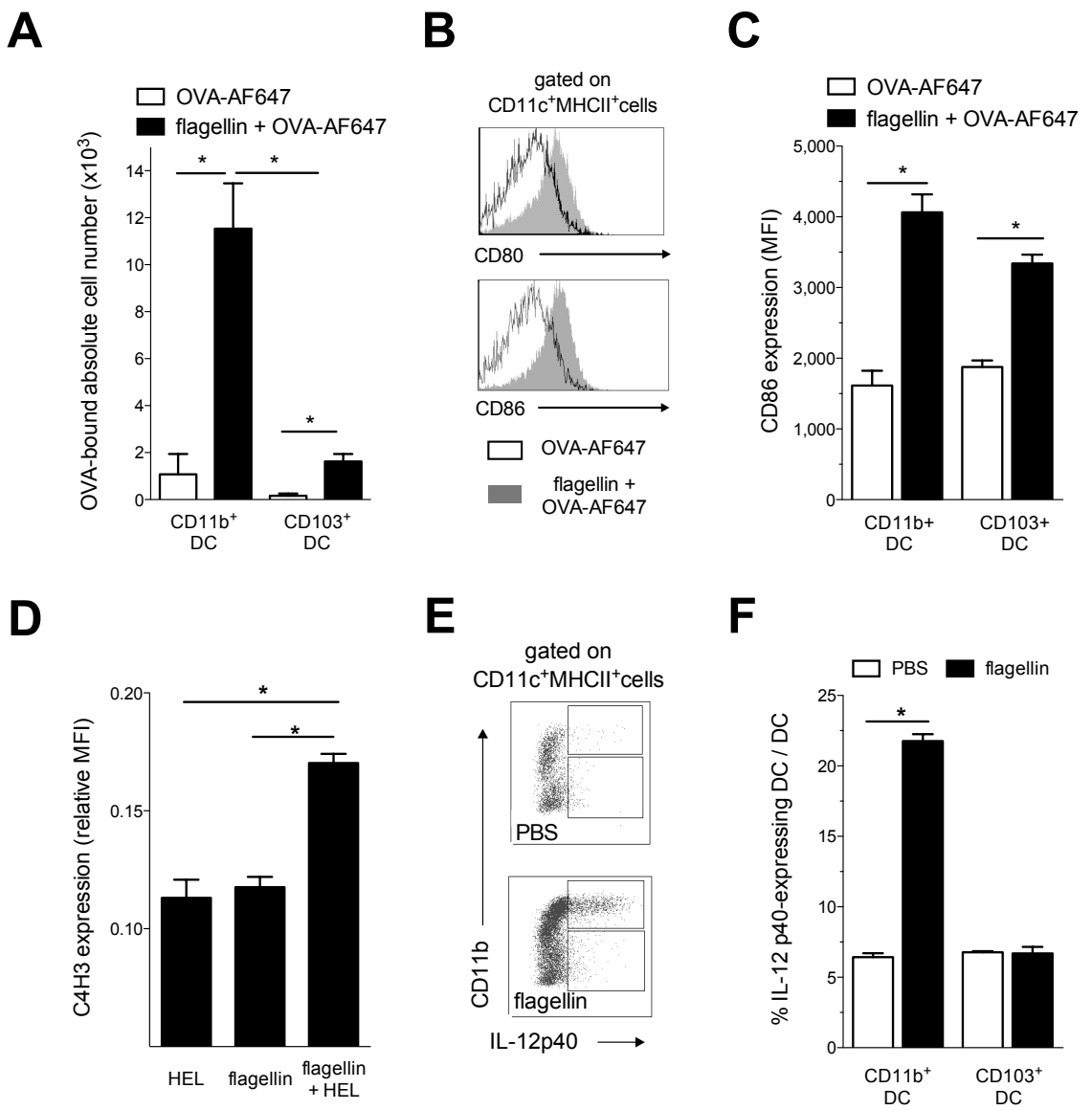


Figure 3: Fougeron *et al.*

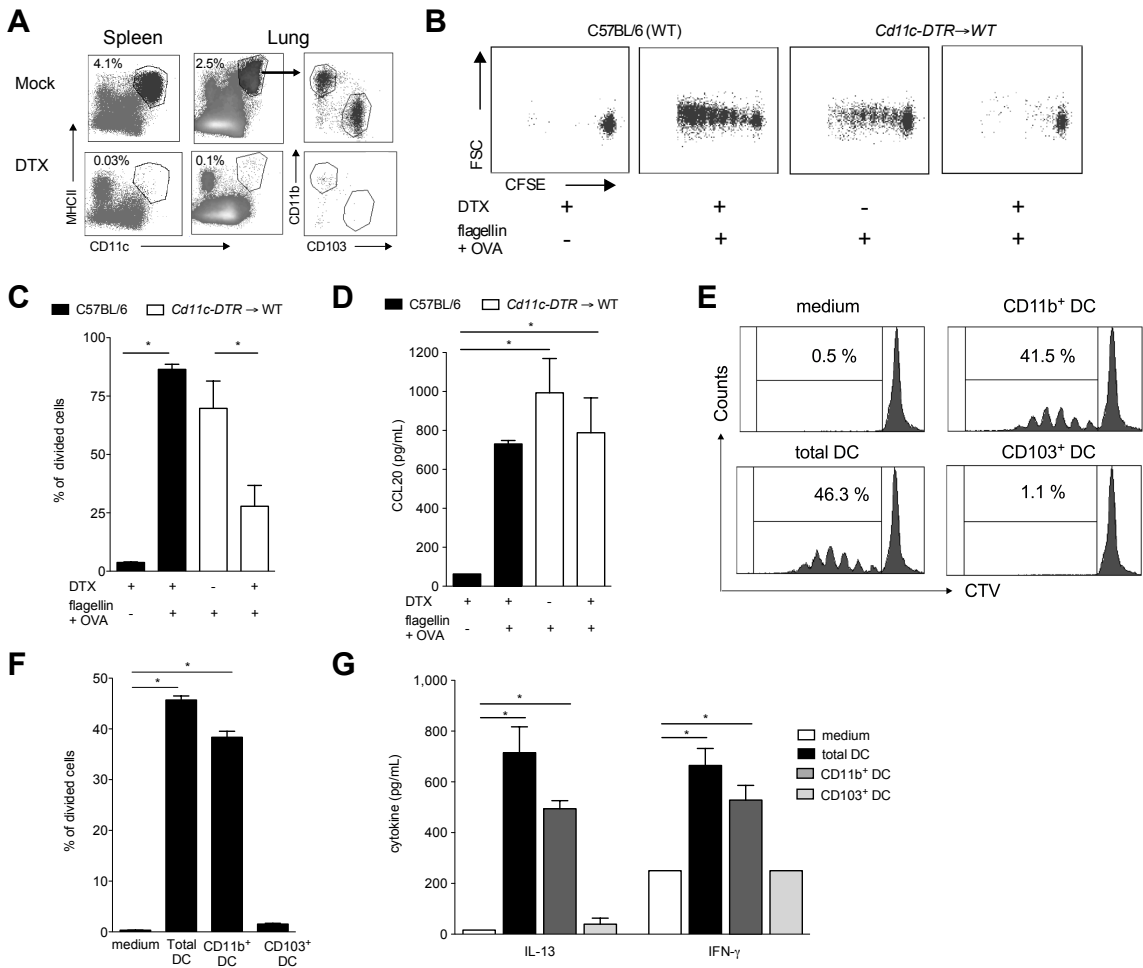


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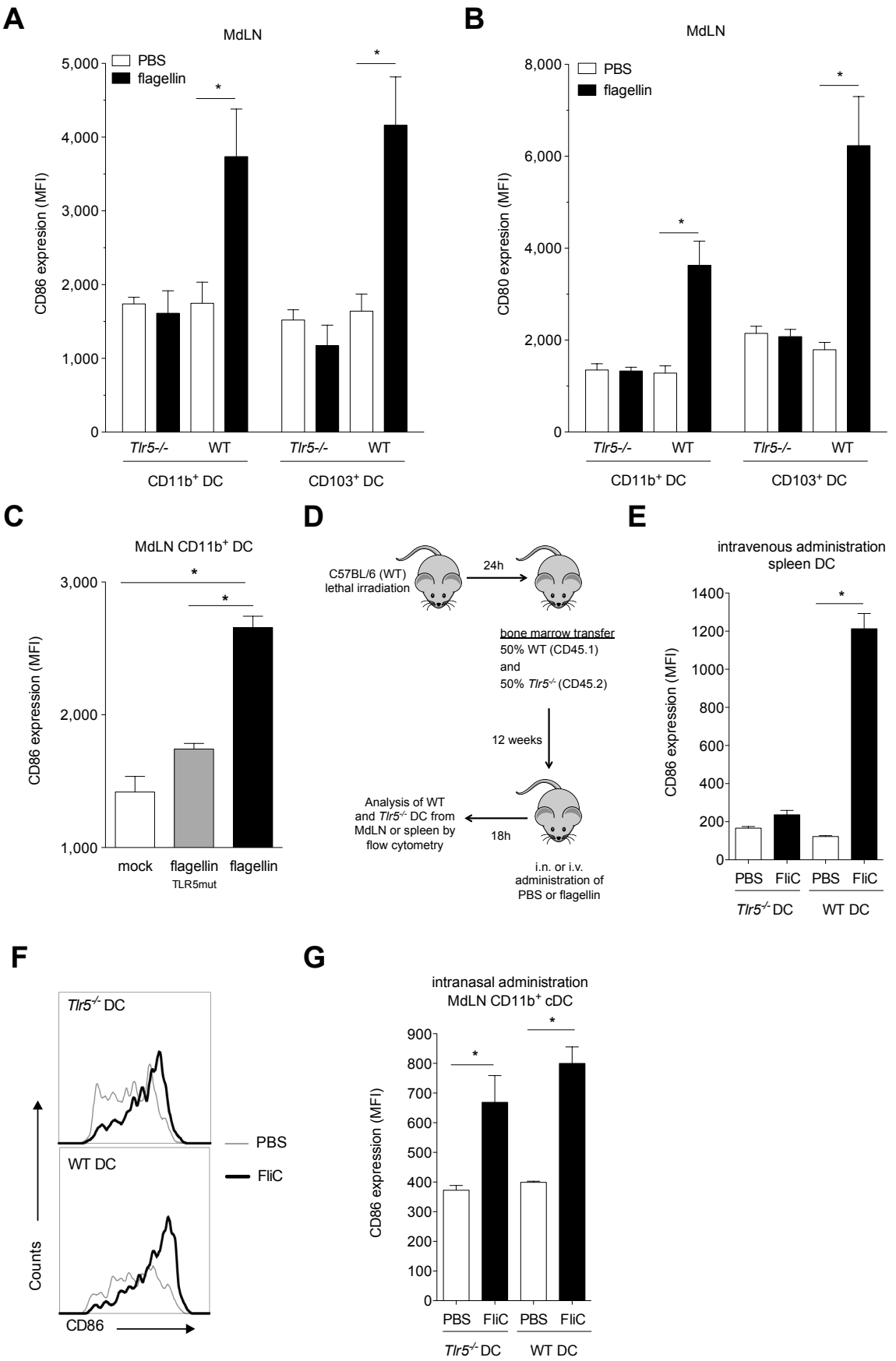


Figure 5 : Fougeron *et al.*

**Conventional dendritic cells promotes the mucosal adjuvant activity of flagellin in the respiratory tract**

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**This file includes Supplementary Materials and Methods, Supplementary Figures S1-S5, and Supplementary References**

## Supplementary Materials and Methods

**Mice.** Six to 10-weeks-old mice *Ccr2*<sup>-/-</sup>, *Tlr5*<sup>-/-</sup> on the C57BL/6 background and NMRI mice were obtained from Taconic or bred in house. Broncho-alveolar lavages (BAL) were collected after the intra-tracheal injection of 1ml PBS with Protease Inhibitor Cocktail (Roche). Lung protein extracts were prepared by homogenizing tissue in Tissue Protein Extraction Reagent (Pierce) supplemented with protease inhibitors as described previously [1, 2].

### Neutrophil and monocyte depletions

Neutrophils were depleted by i.p. injection of anti-Ly6G (NIMP.R14, 200µg), or control isotype HB152 (rat IgG2b) as described previously [3]. Circulating monocytes were depleted by i.v. injection of clodronate liposome or empty liposomes [4]. Immunizations were performed 12h to 24h after the depletions.

**Antigen-specific antibodies.** Immunizations consisted in prime-boost at days 1 and 21 followed by serum sampling at day 28 for OVA-specific antibody titer determination. Levels of OVA-specific IgG were determined by ELISA as previously described [1]. Briefly, MaxiSorp microplates (Nunc) were coated overnight at 4°C with OVA (20µg per well in phosphate buffer 0.2M pH 6.5), washed with PBS/Tween20 0.05%, blocked with PBS/Dry Milk 1% for 1h, and washed again. Serial dilutions of samples were incubated for 1h, washed, and incubated for 1h with HRP-conjugated anti-mouse IgG (Southern Biotechnology Associates). After washing, development was done with 3,3',5,5' tetramethylbenzidine (Becton Dickinson Bioscience) and stopped by addition of H<sub>2</sub>SO<sub>4</sub> and the OD at 450nm was determined. The IgG titer was defined as the reciprocal of the highest sample dilution yielding an absorbance value of 0.1 OD for OVA and was systematically compared with a reference serum.

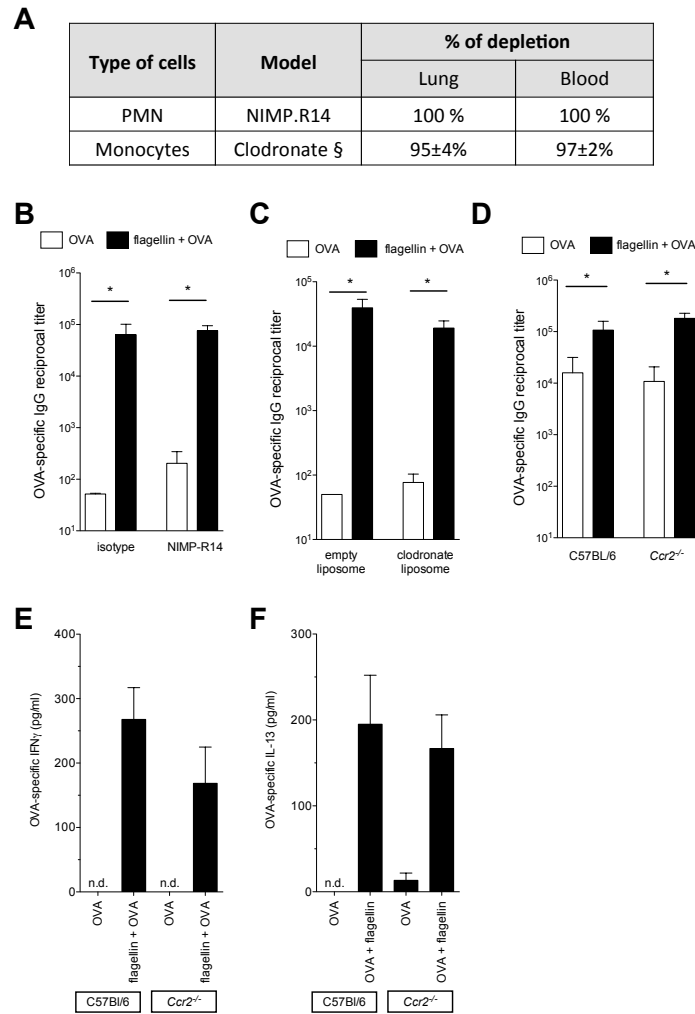
**Cell Culture.** Mouse airway epithelial cells, both mouse tracheal epithelial cells and type II alveolar epithelial cells were differentiated from trachea and lungs of C57BL/6 mice, respectively, as described previously [5-7].

**Gene expression.** Total RNA from whole lung or cultured cells was extracted with Nucleospin RNA kits (Macherey-Nagel). RNA was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems). cDNA was amplified using SYBR Green or TaqMan assays. Relative quantification was performed as described previously [8].

**Antigen-specific cytokine production.** OVA-specific T cell responses were analyzed in mice 7 days after the last immunization. Spleen cells ( $2-5 \times 10^6$ ) were incubated for 72h with RPMI medium supplemented with 5% fetal calf serum ± OVA (1mg/mL) to measure secretion of IL-13 and IFN-γ.

**Microarrays.** Total lung RNA and blood RNA was extracted with the Nucleospin RNA kits (Macherey-Nagel). Lung RNA was processed on Applied Biosystems Mouse Whole Genome Arrays v2.0 and analyzed using the NeONORM method [9]. Blood RNA was processed on Agilent whole mouse genome microarrays and the Rosetta Resolver Biosoftware. Microarray data were MIAME compliant and deposited in the publicly available database:

<http://mace.ihes.fr> with accession number: 2176499768 and <http://www.ncbi.nlm.nih.gov/geo/> with accession number: GSE46421. Gene lists containing group means of expression, p-values and standard fold changes were utilized as input for subsequent bioinformatics analysis. Genes belonging to the same biological functions/pathways were mapped using Ingenuity Pathway Analysis 1.0 (IPA, Ingenuity Systems), Kyoto Encyclopedia of Genes and Genomes (KEGG), and The Immunological Genome Project ([www.immgen.org](http://www.immgen.org)). Heat maps were generated using Gene-E (Broad Institute).



**Figure S1. Monocytes and neutrophils are dispensable for flagellin-mediated adjuvant activity.**

Neutrophils were depleted by i.p. injection of anti-Ly6G (NIMP.R14, 200 $\mu$ g), or control isotype HB152 (rat IgG2b, 200 $\mu$ g). Circulating monocytes were depleted by i.v. injection of clodronate liposome or empty liposomes. Immunizations were performed 12h to 24h after the depletions. (A) C57BL/6 and NMRI (§) mice were injected i.p. with Ly6G-specific depleting antibody NIMP.R14 or isotype HB152 (control), or i.v. with clodronate liposomes or empty liposomes (control). The percentage of depletion was measured with respects to control mice. Neutrophils (CD45<sup>+</sup>lin<sup>neg</sup>CD11b<sup>high</sup>Ly6C<sup>+</sup>Ly6G<sup>+</sup>) and monocytes (CD45<sup>+</sup>lin<sup>neg</sup>CD11b<sup>high</sup>Ly6C<sup>high</sup>Ly6G<sup>neg</sup>) were counted in the lung and in the blood after 12h to 24h. (B) NIMP.R14-depleted and isotype-treated C57BL/6 mice (n=7-8) were vaccinated i.n. at day 0 and day 21 either with ovalbumin (OVA) or flagellin plus OVA. OVA-specific IgG titer was determined in the serum at day 28. (C) NMRI mice (n=5-8) were injected i.v. with clodronate liposomes or empty liposomes and immunized i.n. with OVA or flagellin plus OVA. OVA-specific IgG titer was determined in the serum at day 21. (D-F) C57BL/6 and *Ccr2*<sup>-/-</sup> animals (n=8-9) (C) and (D) were vaccinated i.n. at day 0 and day 21 with OVA or flagellin plus OVA. OVA-specific IgG titers were measured by ELISA at day 28 (D). T cell secretion assays were performed on splenocytes stimulated with OVA for 3 days (E-F). Data are expressed as the mean  $\pm$  SEM and are representative of 1-2 experiments. Statistical significance (\**p*<0.05) was determined using the Mann-Whitney test.

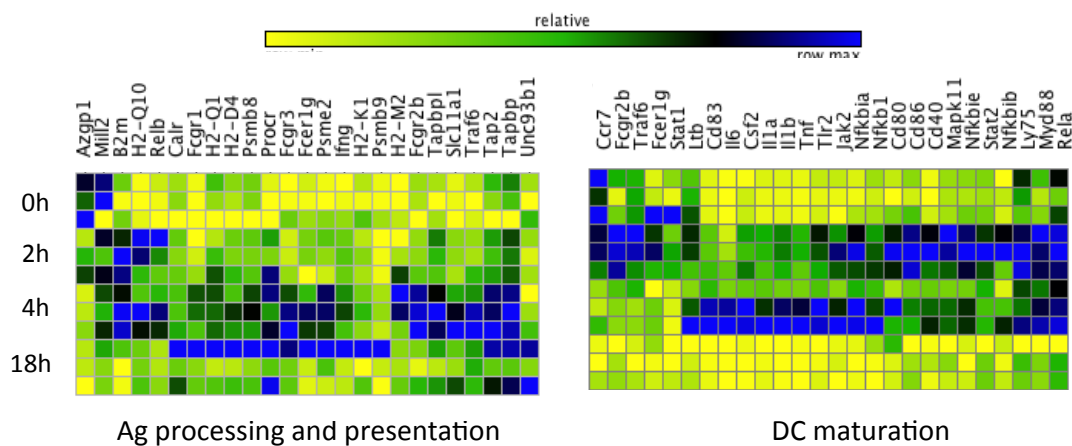


**A**

Lung canonical pathways	-log <sub>10</sub> (p value)		
	2h	4h	18h
Granulocyte Adhesion and Diapedesis	24.77	17.68	9.96
IL-10 Signaling	13.14	16.42	5.29
Acute Phase Response Signaling	13.21	14.69	5.6
Dendritic Cell Maturation	9.14	13.58	2.84
Activation of IRF by Cytosolic Pattern Recognition Receptors	5.86	13.74	0.87
TREM1 Signaling	11.80	10.38	2.82
IL-6 Signaling	10.09	9.89	2.65
NF-κB Signaling	12.18	9.21	2.58

**B**

Lung biological functions	-log <sub>10</sub> (p value)		
	2h	4h	18h
Function of leukocytes	54.67	60.87	15.09
Migration of blood cells	55.32	49.69	n.d.
Inflammatory response	53.46	49.34	18.75
Cell movement	46.32	34.44	15.79
Infiltration of cells	43.66	36.33	16.35
Function of antigen presenting cells	30.84	32.56	6.35
Adhesion of immune cells	32.89	33.12	14.12
Activation of antigen presenting cells	16.65	16.88	9.41

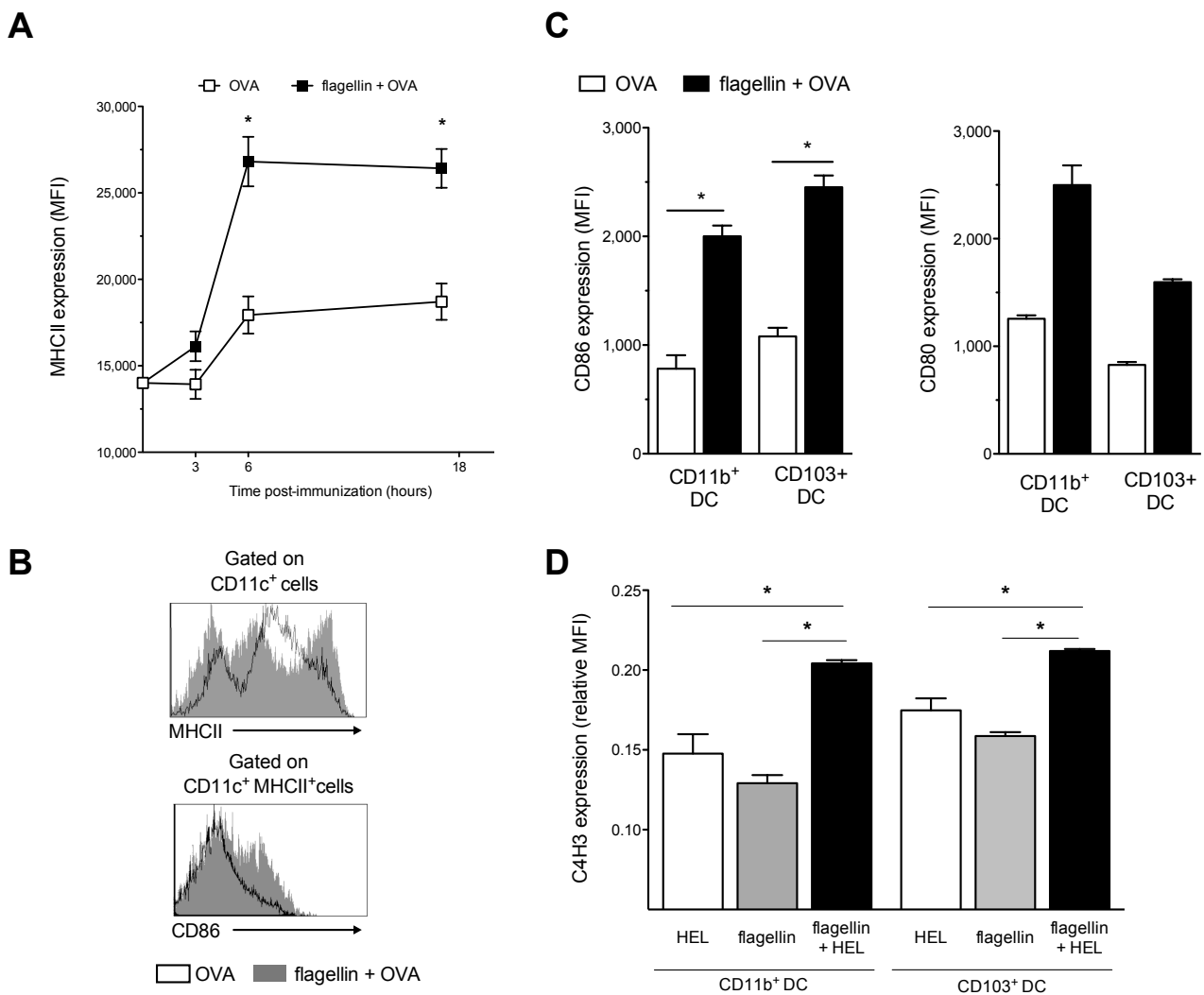
**C****D**

Blood canonical pathways	-log <sub>10</sub> (p value)		
	2h	4h	18h
IL-8 Signaling	1.25	5.54	0.55
Role of NFAT in Regulation of the Immune Response	n.d.	5.48	0.25
Superpathway of cholesterol Biosynthesis	0.70	0.63	5.43
JAK/Stat Signaling	0.35	4.64	0.27
CXCR4 Signaling	0.46	4.00	n.d.
IL-10 Signaling	n.d.	3.25	n.d.
Erythropoietin Signaling	0.37	3.19	n.d.
Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes	0.77	2.92	n.d.

**E**

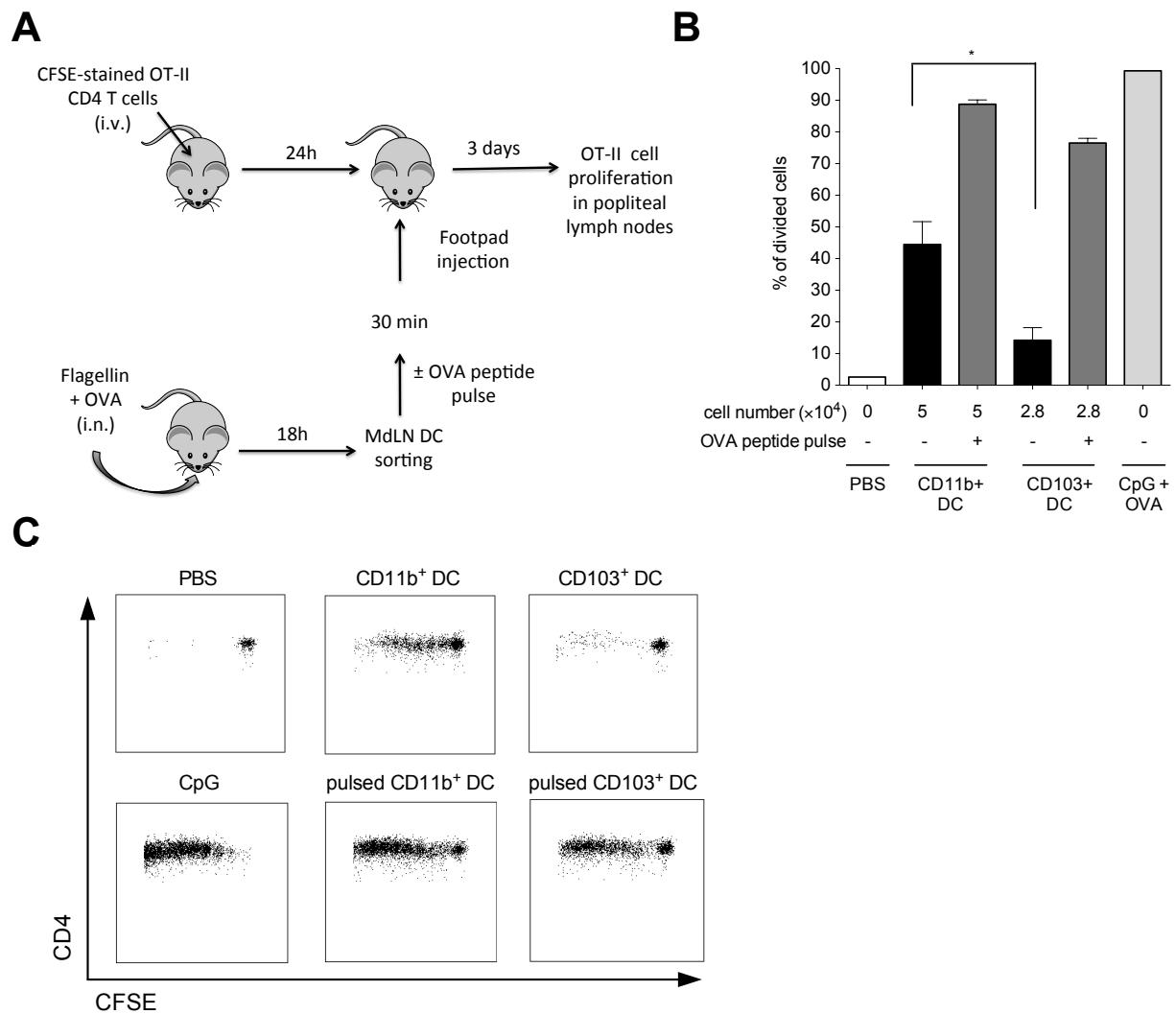
Blood biological functions	-log <sub>10</sub> (p value)		
	2h	4h	18h
Function of leukocytes	n.d.	13.76	n.d.
Migration of blood cells	n.d.	n.d.	n.d.
Inflammatory response	n.d.	8.48	n.d.
Cell movement	4.29	5.62	2.53
Infiltration of cells	n.d.	5.39	n.d.
Function of antigen presenting cells	n.d.	6.77	n.d.
Adhesion of immune cells	n.d.	5.04	n.d.
Activation of antigen presenting cells	n.d.	3.72	n.d.

**Figure S2. The flagellin-specific lung but not blood response displays specific features of DC maturation.** C57BL/6 mice (n=3-5 per group) were treated i.n. with flagellin for 2h, 4h, and 18h. Lung (A-C) and blood (D-E) whole-genome microarrays were analyzed. Differentially expressed genes with absolute fold change of expression > 2 relative to controls (0h) were used for analysis. (A-D, B-E) IPA analysis of microarray data. The significance is expressed as “-log(p-value)”. (A, D) Main canonical pathways upregulated by flagellin in lung (A) and blood (D). (B, E) Main biological functions upregulated by flagellin in lung (B) and blood (E). (C) Heat maps of KEGG molecular functions and cell signatures established according to the Immgen database of lung tissue with respect to DC functions. Heat maps are based on average linkage method and Pearson Correlation as distance metric.



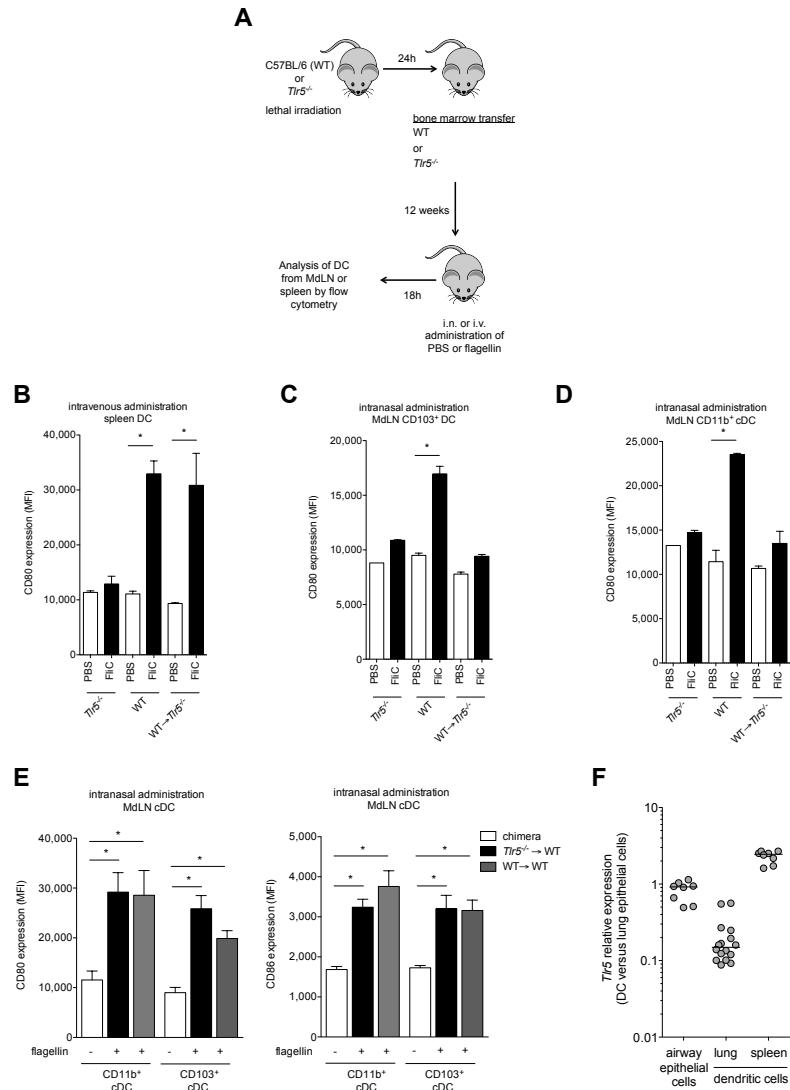
**Figure S3. Intranasal administration of flagellin elicits the maturation of lung dendritic cells.**

C57BL/6 (n=3-4) were immunized i.n. with ovalbumin OVA or flagellin and OVA. Lungs were sampled at indicated time for flow cytometry analysis. Dendritic cells were defined as  $lin^{neg}CD45^{+}CD11c^{+}MHCII^{+}CD11b^{+/-}CD103^{+/-}$ . (A) Expression of MHCII by lung total CD11c<sup>+</sup> cells over time. (B) Representative histograms of MHCII and CD86 expression by the indicated population in the lung. (C) Functional activation of lung CD11b<sup>+</sup> and CD103<sup>+</sup> DCs (CD86 and CD80 expression). Data are presented as the mean fluorescence intensity. (D) C3H/HeN animals (n=4) were immunized i.n. with either hen egg lysozyme (HEL), flagellin, or flagellin and HEL, and lungs were sampled 18h post administration. Antigen presentation by lung CD11b<sup>+</sup> and CD103<sup>+</sup> DCs was assessed with C4H3 antibody that detects HEL peptide loaded on I-A<sup>k</sup>. Fluorescence of the C4H3 antibody was normalized against the I-A<sup>k</sup> fluorescence.



**Figure S4. Mediastinal lymph node CD11b<sup>+</sup> dendritic cells from intranasally immunized animals present antigen to CD4 lymphocytes.**

(A) Schematic representation of the protocol used for *ex vivo* antigen presentation assay. Naïve C57BL/6 mice (n=4 per group) were adoptively transferred with CFSE-labeled ovalbumin (OVA)-specific OT-II CD4<sup>+</sup> T cells 24h before immunization. (ii) C57BL/6 mice (n=16) were immunized i.n. with flagellin and OVA and the mediastinal lymph nodes (MdlN) were sampled 18h later. DCs (lin<sup>neg</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>) were sorted as CD11b<sup>+</sup> and CD103<sup>+</sup> DC subsets and injected in the footpad of adoptively transferred recipient animals. With respects to their relative number in the MdlN,  $5 \times 10^4$  CD11b<sup>+</sup> or  $2.8 \times 10^4$  CD103<sup>+</sup> DC were respectively injected in recipient mice. In addition,  $5 \times 10^4$  CD11b<sup>+</sup> or  $2.8 \times 10^4$  CD103<sup>+</sup> DC loaded in vitro with OVA peptide (323-339) (denominated ‘pulse’) and CpG + OVA footpad immunization were used as positive controls. (B) Proliferation of CFSE-labeled OT-II T cells in the popliteal lymph nodes was analyzed 3 days after the injection (C) Representative dot plots of CFSE dilution of transferred OT-II cells induced by DCs or control treatment. Data are expressed as the mean  $\pm$  SEM and are representative of 1-2 experiments. Statistical significance (\*p<0.05) was determined using the Mann-Whitney test.



**Figure S5. TLR5 expression in airway dendritic cell is not required for response to intranasal flagellin.**

(A) Experimental strategy of bone marrow chimera. Bone marrow from *Tlr5*<sup>-/-</sup> or WT animals were injected into lethally irradiated *Tlr5*<sup>-/-</sup> or WT mice. Three months after engraftment (>95% reconstitution of DC compartment), chimera mice (n=3-5) were immunized with flagellin by intravenous (B) or intranasal (C-E) route. The maturation of spleen DC (B) or mediastinal lymph node (MdlN) DC (C-E) was assessed 18h after PBS or flagellin administration by flow cytometry analysis. Dendritic cells were defined as lin<sup>neg</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>CD11b<sup>+/</sup>CD103<sup>+/</sup>. (B) Quantitative analysis of CD80 expression by splenic DC in response to intravenous immunization. (C-E) Quantitative analysis of CD80 and CD86 expression by MdlN cDCs. (F) Total CD11c<sup>+</sup>MHCII<sup>+</sup> DC were sorted from lung or spleen of C57BL/6 mice. Airway epithelial cells were differentiated from mouse trachea and lung tissues. *Tlr5* mRNA levels were determined by quantitative PCR. Levels of mRNA in airway epithelial cells were arbitrarily set to 1 and used to calculate relative gene expression in spleen DC and lung DC. Data represent the mean ± SEM and are representative of 1-3 experiments (\*p<0.05).

## Supplementary references

- [1] Nempont C, Cayet D, Rumbo M, Bompard C, Villeret V, Sirard JC. Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity. *J Immunol*. 2008;181:2036-43.
- [2] Van Maele L, Fougeron D, Janot L, Didierlaurent A, Cayet D, Tabareau J, et al. Airway structural cells regulate TLR5-mediated mucosal adjuvant activity. *Mucosal Immunol*. 2014;7:489-500.
- [3] Munoz N, Van Maele L, Marques JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal administration of flagellin protects mice from *Streptococcus pneumoniae* lung infection. *Infect Immun*. 2010;78:4226-33.
- [4] Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods*. 1994;174:83-93.
- [5] Messier EM, Mason RJ, Kosmider B. Efficient and rapid isolation and purification of mouse alveolar type II epithelial cells. *Experimental lung research*. 2012;38:363-73.
- [6] Crotta S, Davidson S, Mahlakoiv T, Desmet CJ, Buckwalter MR, Albert ML, et al. Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS Pathog*. 2013;9:e1003773.
- [7] You Y, Richer EJ, Huang T, Brody SL. Growth and differentiation of mouse tracheal epithelial cells: selection of a proliferative population. *American journal of physiology Lung cellular and molecular physiology*. 2002;283:L1315-21.
- [8] Van Maele L, Carnoy C, Cayet D, Songhet P, Dumoutier L, Ferrero I, et al. TLR5 signaling stimulates the innate production of IL-17 and IL-22 by CD3(neg)CD127+ immune cells in spleen and mucosa. *J Immunol*. 2010;185:1177-85.
- [9] Noth S, Brysbaert G, Benecke A. Normalization using weighted negative second order exponential error functions (NeONORM) provides robustness against asymmetries in comparative transcriptome profiles and avoids false calls. *Genomics Proteomics Bioinformatics*. 2006;4:90-109.