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Running title: TLR5-dependent lung inflammatory response

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Abstract

Bacterial products (such as endotoxins and flagellin) trigger innate immune responses through toll-like receptors (TLRs). Flagellin-induced signalling involves TLR5 and MyD88 and, according to some reports, TLR4. Whereas epithelial and dendritic cells are stimulated by flagellin *in vitro*, the cells' contribution to the *in vivo* response is still unclear. Here, we studied the respective roles of radioresistant and radiosensitive cells in flagellin-induced airway inflammation in mice. We found that intranasal delivery of flagellin elicits a transient change in respiratory function and an acute, pro-inflammatory response in the lungs, characterized by TLR5- and MyD88-dependent chemokine secretion and neutrophil recruitment. In contrast, TLR4, CD14 and TRIF were not essential for flagellin-mediated responses, indicating that TLR4 does not cooperate with TLR5 in the lungs. Respiratory function, chemokine secretion and airway infiltration by neutrophils were dependent on radioresistant, TLR5-expressing cells. Furthermore, lung haematopoietic cells also responded to flagellin by activating TNFα production. We suggest that the radioresistant lung epithelial cells are essential for initiating early, TLR5-dependent signalling in response to flagellin and thus triggering the lung's innate immune responses.

Introduction

Upon challenge with respiratory pathogens, the airways rapidly develop a pro-inflammatory response initiated by environmental agents including microbe-associated molecular patterns (MAMP) such as Gram-negative bacteria lipopolysaccharides (LPS or endotoxins). This pro-inflammatory reaction plays an important role in the innate defence against respiratory pathogens [1, 2]. Physiological changes induced by endotoxin inhalation include pulmonary function and bronchoconstriction`, cytokine and chemokine production`, polymorphonuclear neutrophil (PMN) recruitment`, increased permeability of the alveolar epithelium and the associated endothelium [3-6]. Toll-like receptor (TLR) 4 [7], together with MD-2, LPS-binding protein (LBP) and CD14 detect endotoxins and play a critical role in the initiation of the pulmonary response to systemic and mucosal endotoxin administration [5, 8-10]. The pulmonary inflammation to endotoxin involves TLR4 signalling in both the parenchyma cells like endothelial and epithelial cells and the haematopoietic cells, i.e. monocytes, dendritic cells and neutrophils [11]. In addition, TLR4 signalling contributes to the development and progression of chronic respiratory disease including asthma [12-16].

Flagellin is the major constituent of flagella from Gram-negative and Gram-positive bacteria. TLR5 has been identified as the main pattern recognition receptor required for flagellin-mediated immune signalling [17]. TLR5 signalling depends on MyD88 and results in activation of MAPKs, nuclear translocation of NF-κB and production of various proinflammatory cytokines and chemokines [18]. Moreover, a previous report found that TLR5 cooperates with TLR4, suggesting that TLR4 deficiency will affect flagellin-mediated responses [19]. A common stop codon polymorphism in TLR5 is associated to both increased susceptibility to pneumonia caused by the flagellated bacteria *Legionella pneumophila* and resistance to Crohn's disease, indicating that TLR5 signalling is relevant for mucosal immunity [20, 21]. With TLR5-deficient

mice, the critical role of TLR5 in mucosal inflammatory responses was established since deletion of *Tlr5* gene alters response to respiratory, intestinal and urinary tract infections as well as mucosal homeostasis [1, 22-26]. Parenchyma and haematopoietic cells play a different role in TLR5-dependent mucosal immunity depending on the tissue studied. In the gut, dendritic cells of the lamina propria, i.e. haematopoietic cells, have been shown to be instrumental in TLR5 signalling [23]. Using chimeric mice with wild type (WT) or *Tlr5 -/-* bone marrow (BM), Feuillet *et al* observed that TNFα and IL-6 production in bronchoalveolar lavage fluid (BALF) after flagellin administration is dependent on haematopoietic and radioresistant cells while lung neutrophil infiltration mainly required TLR5 competent non-haematopoietic cells [22]. More recently, TNFα production in flagellin-stimulated lung was found to be associated with alveolar macrophages [27].

Here we further investigated the compartmentalization of flagellin-dependent pro-inflammatory responses in the lung. As expected, we found that the response is characterized by lung infiltration by neutrophils, transient local cytokine and chemokine expression. In addition, we showed that flagellin induces alteration of ventilation, a hallmark of respiratory function. We reported that flagellin-mediated activation is abrogated in TLR5- and MyD88-deficient mice but not in TLR4-deficient animals, indicating the sole contribution of TLR5 in flagellin-mediated immunity. Using BM chimeras, we further show that radioresistant cells expressing TLR5, most likely epithelial cells, control respiratory function, chemokine secretion and neutrophil infiltration. Finally, we confirmed that haematopoietic cells, likely alveolar and lung macrophages mediate TNFα production.

Results

Flagellin-induced bronchoconstriction is TLR5-dependent

Since airway exposure to LPS alters ventilatory timing suggesting increase in airway resistance and bronchial responsiveness [11], we assessed whether flagellin, the TLR5 agonist, triggers such a response and whether various TLRs and adaptors contribute to the response. Flagellin was instilled i.n. into mice and the ventilation (PenH) was measured by whole body plethysmography. In wild type (WT) mice, flagellin elicited a rapid PenH increase within 90min, reaching a maximum at 120min and decreasing towards basal level after 4h, while TLR5-deficient mice were unresponsive (Fig. 1A). Trypsin-hydrolysed flagellin did not cause any change in respiratory function, confirming the specificity of the flagellin/TLR5 mediated response. A recent report suggested that TLR5 cooperates with TLR4 [19]. Flagellin-induced airway response was found to be MyD88-dependent, but TRIF-independent (Fig. 1B). We also found that ventilatory function was TLR4- and CD14-independent supporting that the flagellin-induced airway hyperreactivity depends solely on TLR5 signalling (data not shown). Therefore, flagellin promotes the increase of airway resistance and reactivity in a TLR5- and MyD88-dependent manner.

Flagellin-mediated lung PMN infiltration depends on TLR5 signalling

We then investigated whether the alterations of ventilation in response to flagellin in TLR5-deficient mice was associated with effects on lung innate responses as described by Feuillet *et al.* [22]. First, we analysed the total inflammatory cells, PMN and macrophages recruited to the bronchoalveolar space after flagellin administration. While abundant

inflammatory cells recruitment was observed after flagellin administration in the BALF of WT animals *or Tlr4-/-, Cd14-/-* and *Trif-/-* animals, it was absent in *Tlr5-/-* and *MyD88-/-* mice (Fig. 2A-B). Neutrophil infiltration was also characterized in the lung parenchyma by measuring myeloperoxidase (MPO) activity in tissue homogenates. While WT mice showed elevated MPO activity 3h after flagellin administration, *Tlr5-/-* and *Myd88-/-* mice did not display any increased activity similar to saline-treated controls (Fig. 2C and data not shown). In *Tlr4-/-, Cd14-/-* and *Trif-/-*, flagellin promoted MPO increase similarly to WT mice (Fig. 2C). The PMN recruitment was not merely delayed in *Tlr5-/-* and *Myd88-/-* mice as MPO activity did not increase at 24h and 72h (data not shown). Finally, we performed semi-quantitative histological analysis to determine any alterations in the PMN localization in deficient animals (Fig. 3). Tissue sections of flagellin-activated lung showed neutrophil infiltrates in the interstitial and alveolar spaces. The infiltrate was not seen using with trypsin-hydrolysed flagellin (Fig. 3A) and completely absent in flagellin-treated *Tlr5-/-* and *Myd88-/-* mice (data not shown). We confirmed these observations by scoring the infiltration as described in Materials and Methods (Fig. 3B). Therefore, flagellin-mediated transendothelial and transepithelial PMN infiltration into lung parenchyma and bronchoalveolar spaces is TLR5- and MyD88 dependent.

Flagellin response in the lungs mainly depends on TLR5-expressing radioresistant cells

We next examined the respective role of haematopoietic versus stromal/parenchymal cells in the flagellin-induced airway response. For this purpose, we irradiated recipient mice to deplete radiosensitive haematopoietic cells and generated chimera by injection of donor bone marrow (BM) as previously described [28, 29]. Thus, after reconstitution, the lung radioresistant stromal/parenchymal cells were from recipient origin and haematopoietic cells from donor origin.

With respect to ventilatory function, WT animals reconstituted with *Tlr5-/-* (KO) BM (KO→WT) had responses similar to WT animals (Figs. 1A and 4A). Conversely, reconstitution of *Tlr5-/-* mice with WT BM (WT→KO) did not result in any increase of PenH in response to flagellin as observed with TLR5-deficient mice (Figs. 1A and 4A). Therefore, these data show that the alteration of ventilatory function induced by flagellin is essentially due to radioresistant lung cells. Regarding PMN infiltration, KO→WT animals showed a neutrophil influx in BALF similar to the WT control (Fig. 4B). Conversely, WT→KO reconstitution only partially stimulated neutrophil recruitment into the BALF in response to flagellin compared to KO→KO (Fig. 4B). In addition, KO→WT chimera displayed significant levels of MPO activity compared to WT→KO and KO→KO mice where MPO was hardly detected in lung homogenates (Fig.4C). However, we observed a reduced MPO increase in KO→WT compared to controls WT→WT. Histological analyses confirmed the critical role of radioresistant resident cells expressing TLR5 in the PMN recruitment (data not shown). Altogether, these data showed that neutrophil emigration from blood into the lung tissue and alveoli in response to flagellin instillation essentially depends on radioresistant resident cells expressing TLR5. These cells are likely bronchial and/or alveolar epithelial cells since they are known to produce TLR5 and respond in vitro to flagellin by secreting neutrophil-specific chemoattractants like the chemokines CXCL1, CXCL5 and CXL8 [20 , 27, 30 , 31 , 32]. Alternatively, the flagellin-responsive radioresitant cells may be smooth muscle or endothelial cells of the respiratory tract.

We next investigated the contribution of radioresistant cells to the cytokine and chemokine produced in lung homogenates from chimeric mice. We first focused on the chemokines CXCL1 and CXCL5 that are potent PMN chemoattractants. Similar to KO→KO, WT→KO mice produced low levels of CXCL1 within BALF in response to flagellin while WT mice reconstituted with either WT or KO cells produced high levels of CXCL1 (Fig. 5A).

Transcription of *Cxcl5* gene in lung tissue was poorly activated in WT→KO animals like KO→KO control, upon flagellin administration (Fig. 6A). In contrast, KO→WT like WT→WT mice had increased levels of *Cxcl5* mRNA in lungs after flagellin stimulation. These data suggest that the radioresistant resident cells are predominantly responsible for the expression of chemokine genes favouring PMN recruitment in response to flagellin. *Cxcl1* transcription in the lung tissue was upregulated by flagellin treatment in WT→WT but essentially unchanged in KO→KO mice. Interestingly, *Cxcl1* transcription in the whole lung was dependent on both radioresistant resident cells or haematopoietic cells since KO→WT and WT→KO chimera presented comparable increases of *Cxcl1* mRNA levels. The discrepancies between CXCL1 protein in BALF and lung *Cxcl1* mRNA may reflect that the kinetic and the peak of expression is different between the radioresistant and radiosensitive cells or that the secretion is differentially polarized (luminal for radioresistant cells and eventually basal for radiosensitive cells). However, the results support that the chemokines produced by radioresistant compartment are instrumental for the PMN recruitment.

A differential role of parenchymal radioresistant and myeloid cells was found for the expression of the pleiotropic mediators TNFα and IL-6 (Figs. 5 and 6). Analysis of TNFα expression by ELISA and intracellular staining as well as *Tnf* mRNA synthesis showed that WT→KO chimera recapitulated expression of TNFα as WT→WT control and, conversely, that KO→WT and KO→KO mice had no flagellin-dependent TNFα expression (Figs. 5B-D and 6C). Therefore, flagellin-induced TNFα production in lung tissue was clearly dependent on the haematopoietic cells. We also determined that CD11b- Ly6G- parenchymal cells, CD11b+ Ly6G-, CD11b+ Ly6G+ cells were respectively the main producers of TNFα (56%, 22% and 23%). In contrast, IL-6 was produced in BALF when TLR5 is expressed on radioresistant cells (Fig. 5C). However, *Il6* transcription in the lung was variably activated by flagellin both in parenchymal or bone marrow-derived cells that express TLR5 (Fig. 6D).

Finally, TLR5 expression was analyzed by real time qPCR on radioresistant and radiosensitive lung cells, respectively, alveolar epithelial cells and alveolar macrophages as described previously [27]. We found that the relative levels of *Tlr5* mRNA were respectively 0.7310 ± 0.1384 (n=8) and 2.240 ± 0.2402 (n=7), indicating that these two cell types express significant levels of TLR5 and are both competent for TLR5 signalling. This observation supports the compartmentalized effect of TLR5 on both radioresistant and radiosensitive.

Our results suggest that, on the one hand, radioresistant lung epithelial cells are involved in the initiation of inflammatory response by the production of IL-6, but also of chemokines (CXCL1 and CXCL5), which are important for neutrophils recruitment to the lung. On the other hand, we show that TLR5 positive bone-marrow derived-cells are responsible for TNFα secretion.

Discussion

Mucosal tissues are compartmentalized into three regions: the lumen (the external environment), the epithelial barrier and the parenchyma (the lamina propria). How compartmentalization controls mucosal innate immunity is not well understood and needs to be further investigated in order to develop appropriate therapeutic and prophylactic measures. In the mucosa, toll-like receptors (TLRs) induce early signalling dedicated to protective innate immune responses against microorganisms. Here, we studied the respective roles of resident radioresistant and radiosensitive haematopoietic cells in the airway's TLR5-mediated pro-inflammatory response to flagellin in mice. We showed that the airway resistance, chemokine secretion and airway infiltration by neutrophils in response to flagellin depend mainly on the responsiveness of radioresistant lung cells.

In the mucosa, the epithelial cells' prime position at the border between internal and external compartments means that they are the first to encounter pathogens. Hence, in the respiratory tract, bronchial and alveolar epithelial cells with alveolar macrophages are the main sentinels in the detection of microbial signals through innate receptors like the TLRs [33]. A variety of studies have demonstrated that lung and intestinal epithelial cells respond to flagellin by producing PMN-specific chemokines (CXCL1, CXCL5 or CXCL8), cytokines (TNFα and IL-6) and antimicrobial molecules (NO and h-βD2) [18]. These findings agree with TLR5's predominant expression in lung and intestinal epithelial cells; hence, the epithelium is considered to be one of the main TLR5-responsive compartments [34, 35]. Here, we used *Salmonella typhimurium* flagellin and its cognate detector TLR5 as models for investigating the contribution of radioresistant and radiosensitive cells to lung innate immunity. As previously described [22], PMN recruitment was greater in animals in which wild type haematopoietic cells were replaced by *Tlr5-/-* cells (Fig. 4B) - suggesting that *in vivo*, TLR5 signalling in radioresistant (and probably epithelial) cells is prominent in lung responses. Our study further showed a similar

dependence for respiratory function and the secretion of the PMN-specific chemokines CXCL1 and CXCL5 (Figs. 4-5). However, discrimination of compartment contributions by *Cxcl1* transcriptional analyses was less clear-cut; this discrepancy between CXCL1 protein and *Cxcl1* transcripts may represent a difference in the kinetics of transcription-translation-secretion, the nature of compartment analyzed (BALF vs lung), a threshold in effective production of gene product and/or polarized secretion of proteins in our experiments. Consequently, our study reinforces the hypothesis whereby the epithelium has a key role in *in vivo* mucosal, pro-inflammatory processes.

In the literature, there are some divergences on how TLR5 is exposed on epithelial cells [36]. Since TLR5 is active when flagellin is administered intranasally, we assume that apical/luminal signalling induces the epithelial response, as described previously [37]. It has been suggested that this type of epithelial innate mechanism is essential in mucosal defence against *Legionella pneumophila*, *Pseudomonas aeruginosa* and uropathogenic *Escherichia coli* [22, 26, 38]. Furthermore, respiratory pathogens that reach the basolateral side of the epithelial barrier via specific transport mechanisms or following trauma can stimulate either epithelial cells, airway smooth muscle, endothelial cells or any TLR5-expressing haematopoietic cells (such as lung macrophages and dendritic cells). Notably, the intestine's innate response to flagellin relies on dendritic cell activation [1, 23, 39]. This compartmentalization may control the production of pro-inflammatory responses to signals from the gut's microbial flora.

In lung tissue, both non-haematopoietic radioresistant cells (such as epithelial cells) and haematopoietic radioresistant cells (such as alveolar, lung macrophages and dendritic cells) are instrumental in inducing TLR4-dependent innate responses [11, 40]. Literature studies have shown that LPS-induced changes in airway function and PMN recruitment depend on radioresistant cells, while production of cytokines (such as TNFα or IL-12p40) depends on the TLR4/MyD88 pathway in tissue-resident myeloid cells. Here, we also observed that resident

haematopoietic cells participate in TLR5-mediated innate responses to a certain extent, since TNFα production is abolished in WT mice reconstituted with TLR5-deficient bone marrow (Fig. 5B). Following systemic activation, both radiosensitive and radioresistant cells respond to flagellin [41, 42]. Haematopoietic cells are the major source of the pro-inflammatory cytokines TNFα, IL-12 and IL-6, whereas radioresistant cells produce CXCL1, CXCL10 and CCL2. Taken as a whole, these data suggest that radioresistant and radiosensitive cells play distinct roles in the innate response to flagellin in systemic and mucosal models.

In TLR4-mediated lung innate responses, alveolar macrophages are the main LPS target [40]. Interestingly, alveolar macrophages and epithelial cells display similar TLR5 expression levels. Recently, lung macrophages have been identified as the main source of TNFα production following flagellin stimulation [27, 38]. However, the impact of this type of signalling on the host's defence against pathogens remains to be defined. Macrophages have a central role in the maintenance of immunological homeostasis and the host defences. Cross-talk between lung epithelial cells and alveolar macrophages is known to inhibit the latter's inflammatory properties [43, 44]. Although alveolar macrophages are quiescent under steady-state conditions, they can be activated by extrinsic and intrinsic stimuli and then participate in phagocytosis, bacterial killing and tissue wound healing [45, 46]. As described above, epithelial cells also sense bacterial products and activate TLR signalling, which essentially promotes PMN-mediated immunity. The matter of how epithelium and macrophages collaborate on local amplification or sequential activation of innate immunity is still under investigation. Recent observations have revealed that previous exposure to influenza virus determines the functional properties of tissue-residing macrophages in a TLR-dependent manner [38]. Whether other TLR5-expressing haematopoeitic cells like dendritic cells cooperate with epithelium also remains to be further studied. Lastly, all the studies performed to date show that only TLR5 is crucial for the initiation of PMN

recruitment into lungs and the alteration of airway resistance in response to free flagellin; this is not the case for intracellular sensors like IPAF and NAIP5 [47].

Here, we investigated the contribution of TLR4 and the CD14 co-receptor to the pulmonary immune response to flagellin. Previous *in vitro* studies have suggested that TLR4-TLR5 heterodimers detect flagellin and then produce nitric oxide (NO) and IFNβ in a STAT1-dependent manner [19]. The CD14 receptor is also required to potentiate immunity in response to various TLR agonists, in a TLR4-independent process [48-50]. Our results clearly show that TLR4- and CD14-deficient mice respond to flagellin in the same way as WT mice do (Figs 1-3). These data are corroborated by experiments on animals lacking the TRIF adaptor molecule used by TLR4. Hence, the flagellin-mediated lung innate response depends solely on the TLR5 receptor and the MyD88 adaptor.

Airway epithelial cells also produce chemokines (such as CCL20) that attract monocytes/dendritic cells and that may potentiate adaptive immunity in a mucosal adjuvant mechanism [34][51-55]. In contrast, flagellin may directly activate lung dendritic cells [48, 56]. In systemic activation, dendritic cells are directly activated by flagellin in a MyD88-dependent manner [41, 42] and this maturation is crucial for initiation of cellular and humoral adaptive immunity [57]. Flagellin also promotes bystander dendritic cell maturation via release of paracrine mediators from MyD88-competent radioresistant cells [41, 42]. It remains to be seen whether this type of mechanism (combined with *de novo* dendritic cell recruitment by specific chemokines) operates in lung tissue.

Our data reveal that TLR-competent radioresistant cells (probably epithelial cells) are key players in respiratory innate responses to TLR agonists. In conclusion, we believe that understanding the unique role of epithelial TLR signaling and cross-talk with haematopoietic cells will open up new avenues for the manipulation of innate and adaptive mucosal defenses to pathogens.

Materials and Methods

Mice

TLR5-/- [23], MyD88-/- [58], CD14-/- [59], TLR4-/- [60], and TRIF-/- [61] backcrossed at least 10 times on C57BL/6 genetic background as well as control C57BL/6 (CD45.2) or congenic CD45.1 counterpart C57BL/6-Ly5.1 were bred in our animal facility at the Transgenose Institute (CNRS, Orleans). For experiments, adult (6-10 weeks old) animals were kept in sterile isolated ventilated cages. All animal experiments complied with the French Government's ethical and animal experiment regulations.

Flagellin administration and measurement of respiratory function

LPS-depleted flagellin FliC from *Salmonella enterica* Serovar Typhimurium (1µg) produced as described previously [56], LPS (10 µg) from *Escherichia coli* (serotype 055: B5, Sigma St Louis, MO, USA) or saline were given by the aerogenic route using nasal instillation in a volume of 40 µL under light ketamine-xylazine anaesthesia. The LPS concentration contamination as determined using the Limulus assay (Associates of Cape Cod Inc., USA) was less than 10 pg LPS per µg of FliC. When specified, flagellin was treated for 1h at 37°C with trypsin-EDTA 0.017% (Invitrogen, USA) to hydrolyze the protein, followed by heating at 70°C for 1h to inactivate trypsin. As mock control, wild type mice were administered with saline. The airway function was evaluated by whole-body plethysmography [6]. Unrestrained conscious mice were placed in plethysmography chambers (EMKA Technologies, France), and Enhanced respiratory Pause (PenH), measured over 3 to 6 hr. PenH can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves indicating respiratory dysfunction pointing to airway resistance. Increased phase shift correlates with increased respiratory system resistance. PenH is

calculated by the formula PenH = (Te/RT-1) x PEF/PIF, where Te is expiratory time, RT is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow [62].

Bronchoalveolar lavage fluid (BALF)

BALF was collected 24 hr after flagellin or endotoxin administration by cannulating the trachea under deep ketamine-xylazine anaesthesia and washing the lung 4 times with 0.5 mL of saline at room temperature [62]. The lavage fluid was centrifuged 10 min at 2000 rpm 4°C and the supernatant was frozen for cytokine analysis. The cell pellet was suspended in PBS, counted in a haematocytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge (1000 rpm, 10 min). The cells were stained with Diff-Quick (Dade Behring, Marburg, Germany). The supernatant was used for the measurement of cytokine and protein levels. TNF, IL-6 and KC (CXCL1) were measured by enzyme-linked immunosorbent assay (R&D Duoset, Minneapolis, MO).

Microscopy and myeloperoxidase activity (MPO) in lung

After bronchoalveolar lavage and lung perfusion, the mice were sacrificed. The lung were fixed in 4% buffered formaldehyde for standard microscopic analysis, 3 μm sections were stained with haematoxylin and eosin (H&E) [62]. Neutrophil and erythrocyte accumulation in alveoli, disruption of alveolar septae and activation of alveolar epithelial cells were quantified using a semi-quantitative score with increasing severity of changes (0-5) as described previously [6, 63]. Groups of 5-8 mice were included in each experiment and 10 randomly selected high power fields (400 x magnifications) per animal were analyzed.

Lung tissue MPO activity was evaluated as described [10]. In brief, the right heart ventricle was perfused with saline to flush the vascular content and lungs were frozen at -20°C until use. Lung was homogenized by Polytron, centrifuged and the supernatant was discarded. The pellets were suspended in 1 mL PBS containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB) and 5 mM ethylene-diamine tetra-acetic acid (EDTA). Following centrifugation, 50 µL of supernatants were placed in test tubes with 200 µL PBS-HTAB-EDTA, 2 mL Hanks' balanced salt solution (HBSS), 100 µL of o-dianisidine dihydrochloride (1.25 mg/mL), and 100 µL H_2O_2 0.05%. After 15 min of incubation at 37°C in an agitator, the reaction was stopped with 100 µL NaN_3 1%. The MPO activity was determined as absorbance at 460 nm against medium.

Bone marrow transplantation to obtain mixed chimera

Recipient mice underwent a lethal total-body irradiation as reported [28, 29]. Fresh, unseparated bone marrow cells (3 x 10^6 per mouse) were injected into the lateral tail vein of the irradiated recipient mice 24 hr after lethal irradiation (8 gray). The reconstituted mice were used at 3 months after bone marrow transplantation. The degree of chimerism for different haematopoietic-derived cells type was assessed by measuring CD45.1 and CD45.2 surface expression by blood leukocytes and alveolar macrophages in lung tissue. The current protocol yielded more than 96% of reconstitution in the blood; WT mice reconstituted with TLR5-deficient bone marrow presented 90% of CD11b+ CD45.2+ and TLR5-deficent mice reconstituted with WT bone marrow have more than 95 % of CD11b+ CD45.1+ in lung tissue.

Cytokine gene expression.

Total RNA from mouse lungs was extracted with the Nucleospin RNA II kit (Macherey Nagel, Germany) and reverse-transcribed with the High-Capacity cDNA Archive kit (Applied

Biosystems, USA). The resulting cDNA was amplified using SYBR Green-based real-time PCR (Applied Biosystems). The specific primers are CGTCATCCATGGCGAACTG / GCTTCTTTGCAGCTCCTTCGT (*Actb* coding β-actin), CTTGGTTCAGAAAATTGTCCAAAA / CAGGTGCCATCAGAGCAGTCT (*Cxcl1*), TGGATCCAGAAGCTCCTGTGA / ATTCCGCTTAGCTTTCTTTTTGTC (*Cxcl5*), CATCTTCTCAAAATTCGAGTGACAA / CCTCCACTTGGTGGTTTGCT (*Tnf*) and GTTCTCTGGGAAATCGTGGAAA / AAGTGCATCATCGTTGTTCATACA (*Il6*). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined by comparing (a) the PCR cycle thresholds (Ct) for the gene of interest and *Actb* (ΔCt) and (b) ΔCt values for treated and reference group (ΔΔCt), as described previously [56].

Relative expression levels of *Tlr5* mRNA were measured by qRT-PCR and were normalized to 18S RNA level using a standard curve method and the following primers for *Tlr5* (CGCACGGCTTTATCTTCTCC, GGCAAGGTTCAGCATCTTCAA) and 18S rRNA (TTGGCAAATGCTTTCGCTT, CGCCGCTAGAGGTGAAATTC). Results are expressed as geometric mean ± SEM. Alveolar epithelial cells and macrophages were isolated as described previously [27].

Flow cytometry analysis

Lung were minced with scissors and digested in HBSS containing collagenase II (200U/ml), DNase (50U/ml) during 2 hours at 37°c and filtered (100µm). Cells were then washed three times and counted. 10^6 cells isolated from lung or BALF were stained for 30 min, 4°C in 50µl with anti-Ly6G-FITC and anti-CD11b-PerCP Cy5.5 in staining buffer (PBS/BSA 0,5 %). For intracellular staining, cells were permeabilized with Fixation/Permeabilization solution (Perm Wash, Beckton Dickinson) for 20 min at 4°C, and intracellular cytokine TNF was detected using

anti-TNFα-APC during 30 min at 4°C. After extensive washes, cells were analyzed on a Becton Dickinson LSR analyzer. All antibodies were from Becton Dickinson.

Statistical Analysis

Statistical significance was determined by Student *t* test, Mann-Whitney and one-way ANOVA test (Bonferonni) using GraphPad software. *P* values of <0.05 were considered statistically significant.

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Figure Legends

Figure 1: Flagellin-mediated alteration of respiratory function depends on TLR5 and MyD88.

(A) The indicated mice were administered intranasally with saline (mock), flagellin (1µg) or trypsin-hydrolysed flagellin (1 µg equivalent) and then monitored by plethysmography. Respiratory function (PenH) was measured from 60 min to 360 min. The ventilatory function is expressed as mean +/- SEM of PenH. Native flagellin induced PenH in C57BL/6 mice (black squares) but not in *Tlr5* -/- animals. Saline and trypsin-hydrolysed flagellin did not increase PenH. (B) PenH in the indicated mice is expressed as area under the curve (AUC) measured from 60 to 360 minutes after flagellin (1µg) administration. Data are from one representative experiment of three (n=4 mice per group). Statistical analysis was performed using Student *t* test (*p<0.05 ; **p<0.01).

Figure 2: TLR5- and MyD88-dependent neutrophil recruitment in the lung of flagellin-stimulated mice.

The indicated mice were administered intranasally with saline (mock) or flagellin (1µg). (A, B) BALF was collected after 24 h and (A) the total number of cells and (B) the cellular composition determined. (C) Lungs were lysed 3h after flagellin administration and MPO activity measured in the lysates to assess neutrophil infiltration in the lung tissue. Data are from one representative experiment of three and are expressed as mean +/- SD (n=4 mice per group). Statistical analysis was performed using Student *t* test (*p<0.05 ; **p<0.01 ; ***p<0.001).

Figure 3: Histological analysis of neutrophil infiltration in lungs of flagellin-stimulated wild type and TLR-deficient animals.

Animals were administered intranasally with saline (mock), flagellin (1µg), trypsin-treated flagellin (1µg equivalent), or LPS ((AU: concentration of LPS?)). The lungs of C57BL/6, *Tlr4-/-*, *Tlr5-/-*, *Cd14-/-*, *Myd88-/-* and *Trif-/-* mice were analysed by microscopy 24h later. (A) Representative histology in C57BL/6 animals (magnification x200 and x400 in inserts, HE coloration). (B) The PMN infiltration in flagellin-treated mice was assessed by a semi-quantitative score. The neutrophil infiltration observed in C57BL/6, *Tlr4-/-*, *Cd14-/-*, and *Trif-/-* mice was absent in *Tlr5-/-* and *Myd88-/-* mice. The same pattern was observed for the disruption of the microarchitecture and the activation of alveolar epithelia (data not shown). Data are from one representative experiment out of three and are expressed as mean values +/- SD (n=4 mice per group; Statistical analysis was performed using Student *t* test (**p<0.01 ; ***p<0.001).

Figure 4: Major role of radioresistant cells in flagellin-specific respiratory function and neutrophil infiltration.

Chimeric mice generated by injection of bone marrow from C57BL/6 (WT) or TLR5-deficient (KO) mice into KO or WT mice respectively and 1µg flagellin administered intransally. (A) Respiratory function (PenH) was measured from 60 min to 180 min. (B) Cell composition of BALF and (C) MPO activity in lung homogenates 4h after flagellin administration. Data are expressed as mean +/- SD (n=4 mice per group; Student *t* test *p<0.05 ; **p<0.01 ; ***p<0.001). ((AU: Are the data from one representative experiment out of three?))

Figure 5: Cell compartmentalization of TLR5-dependent lung production of chemokines and cytokines

Chimeric mice generated by injection of bone marrow from C57BL/6 (WT) or TLR5-deficient (KO) mice into KO or WT mice respectively and 1µg flagellin was administered intranasally.

Analysis was performed 4h later. (A) CXCL1 production in lung homogenates. (B) TNFα and (C) IL-6 production in BALF. (D) Intracellular staining of TNFα in lung cells. (A-C) Data are expressed as mean +/- SD (n=4 mice per group. (D) Diamonds represent data from individual mice ((AU: OK?)). One way ANOVA test *p<0.05 ; **p<0.01 ; ***p<0.001; n.d., not determined. ((AU: Are the data from one representative experiment out of three? Was ANOVA used for all panels in Fig 5?))

Figure 6: Chemokine and cytokine gene transcription in the lungs of chimeric mice

Chimeric mice generated by injection of bone marrow from C57BL/6 (WT) or TLR5-deficient (KO) mice into KO or WT mice respectively and 1µg flagellin administered intransally. Analysis was performed 4h later. Relative (A) *Cxcl5*, (B) *Cxcl1,* (C) *Tnf,* and (D) *Il6* mRNA levels. Values were determined by RT-PCR using KO>KO as the calibrating condition. Data are expressed as mean +/- SD (n=4 mice per group; Mann-Withney test. *p<0.05; **p<0.01).

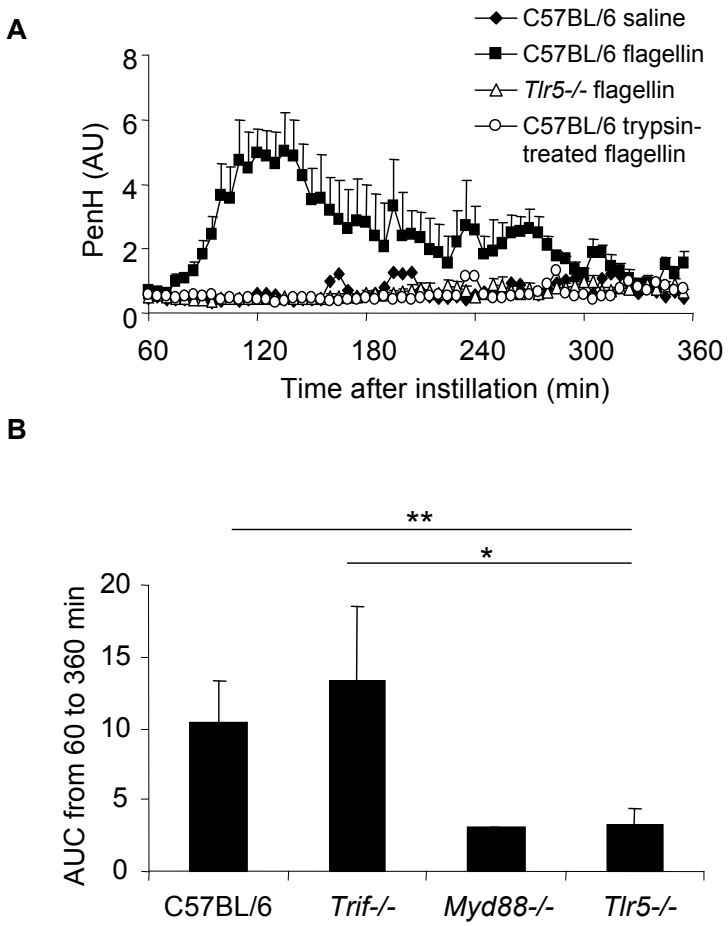
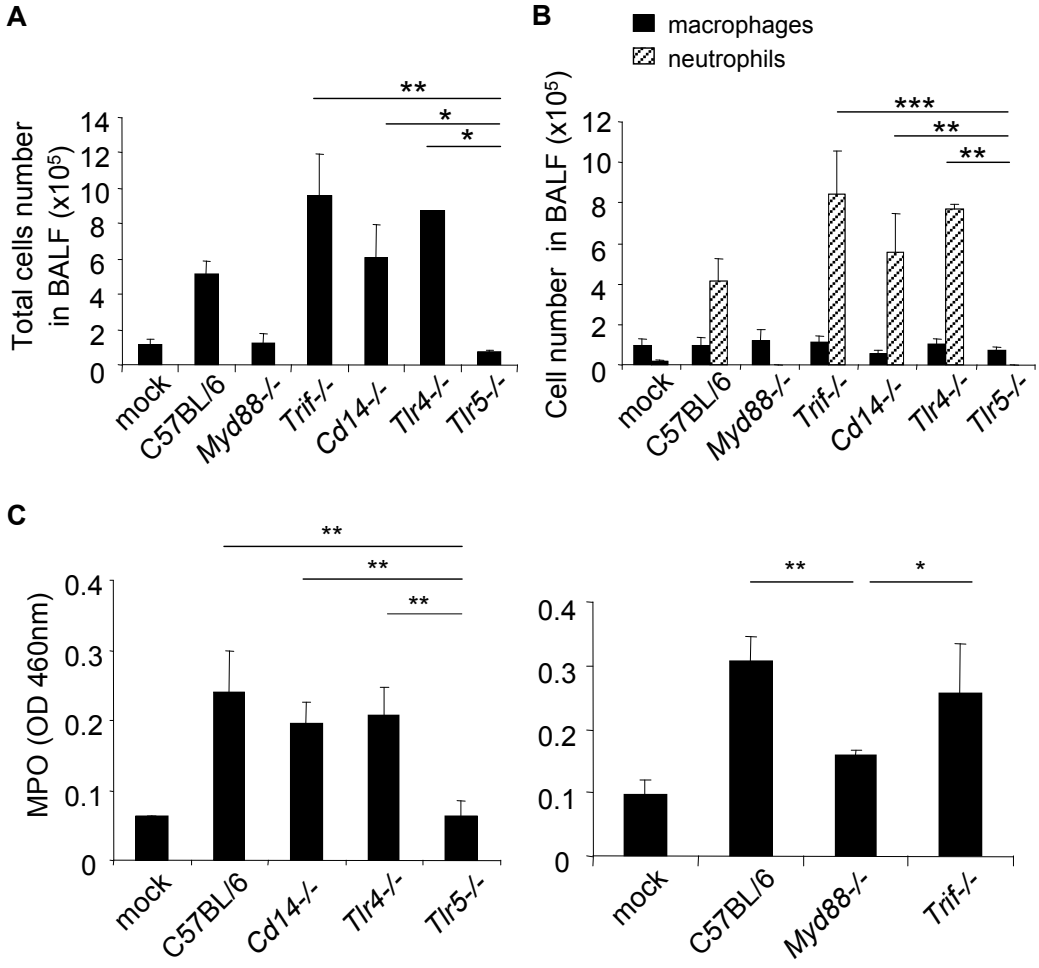


Figure 1 Janot *et al.*



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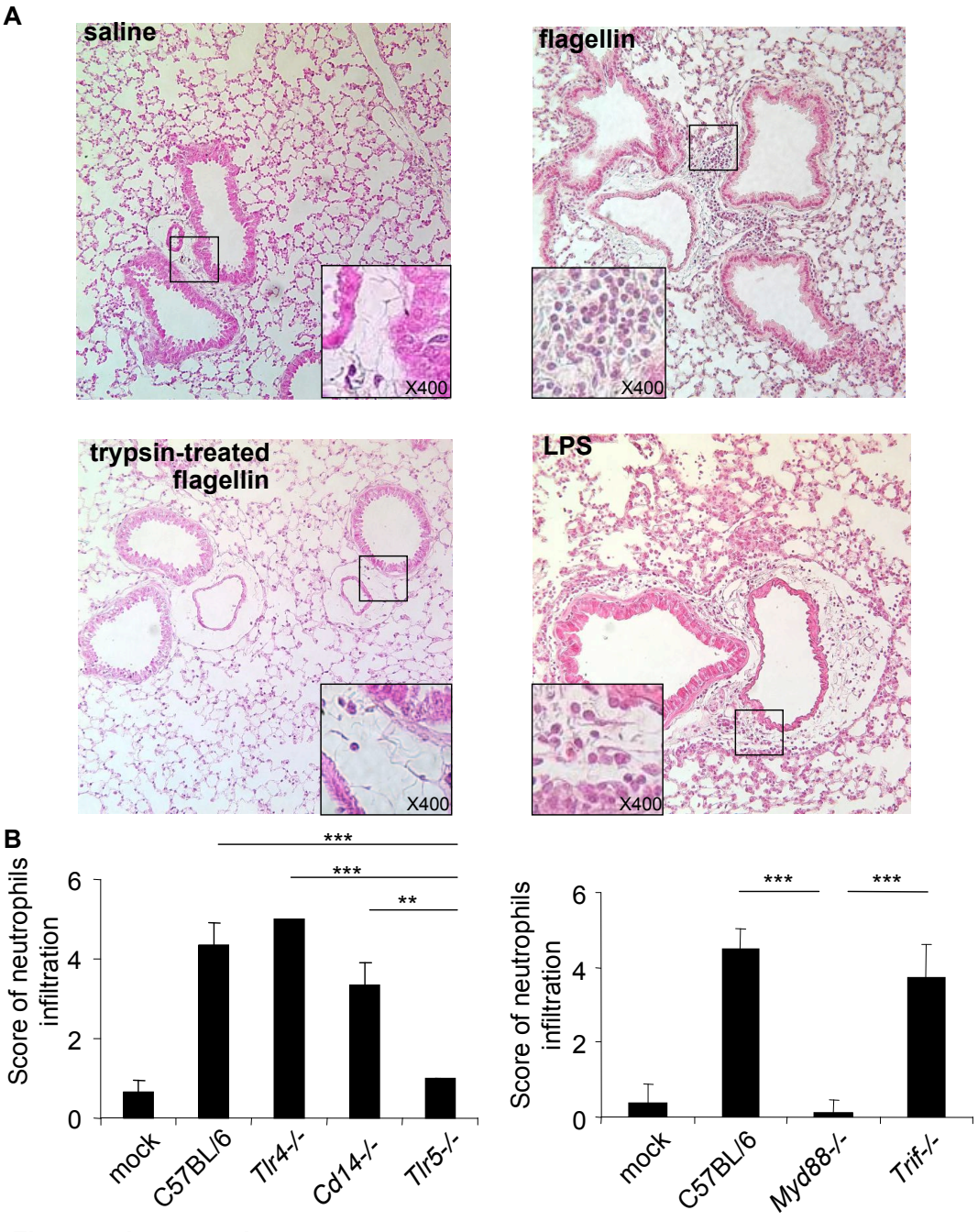

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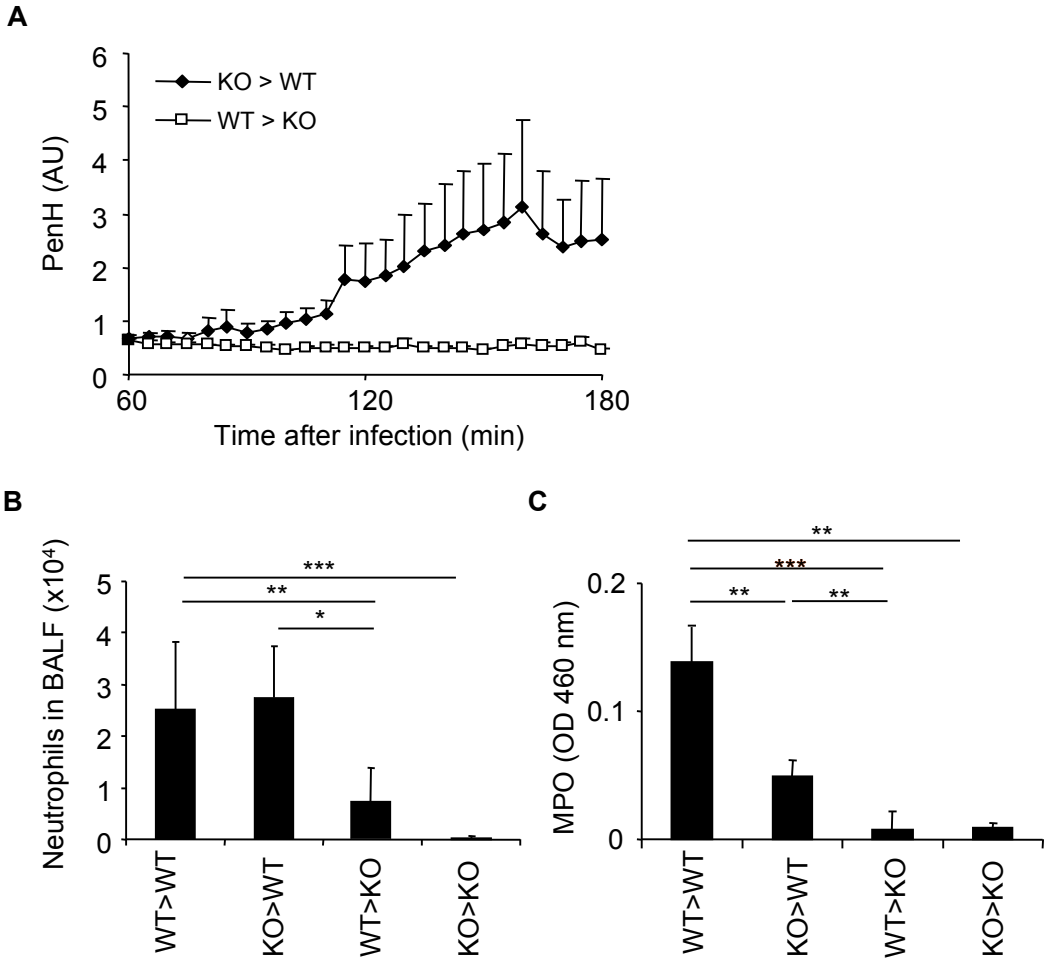

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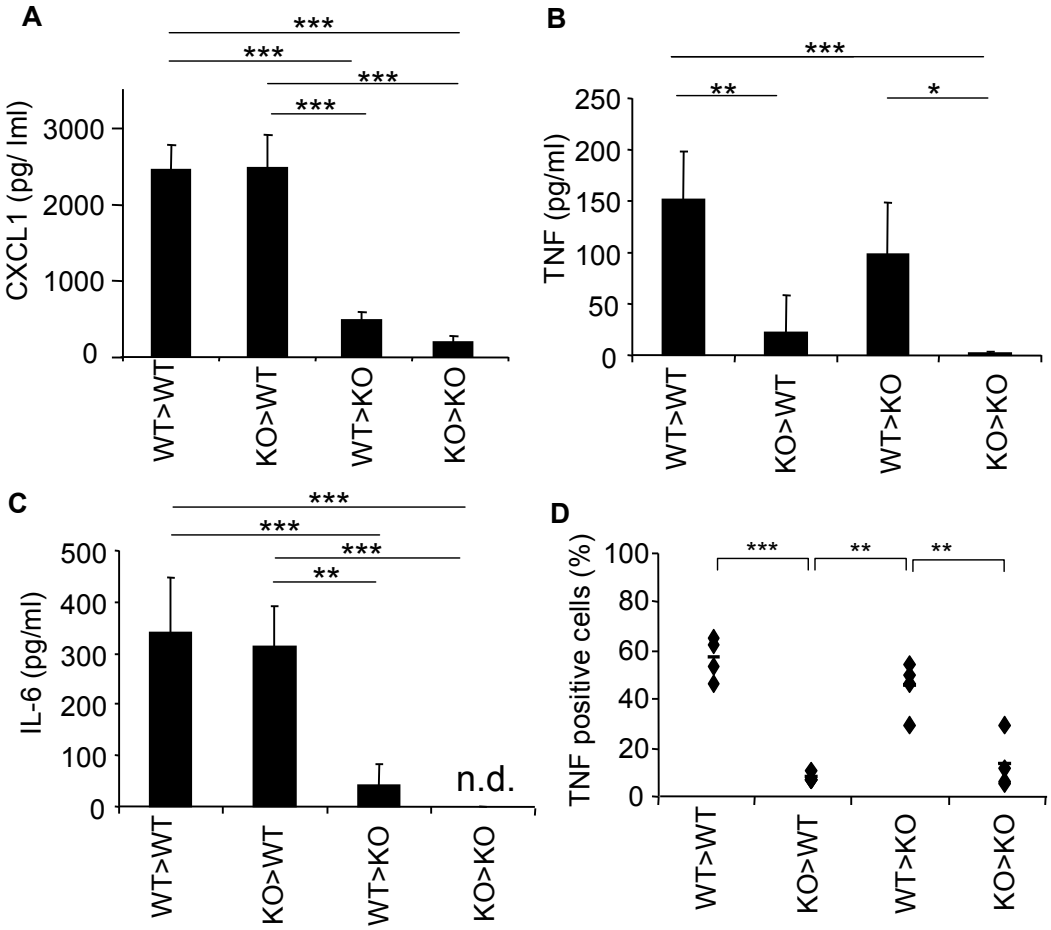

Figure 5 Janot *et al.*

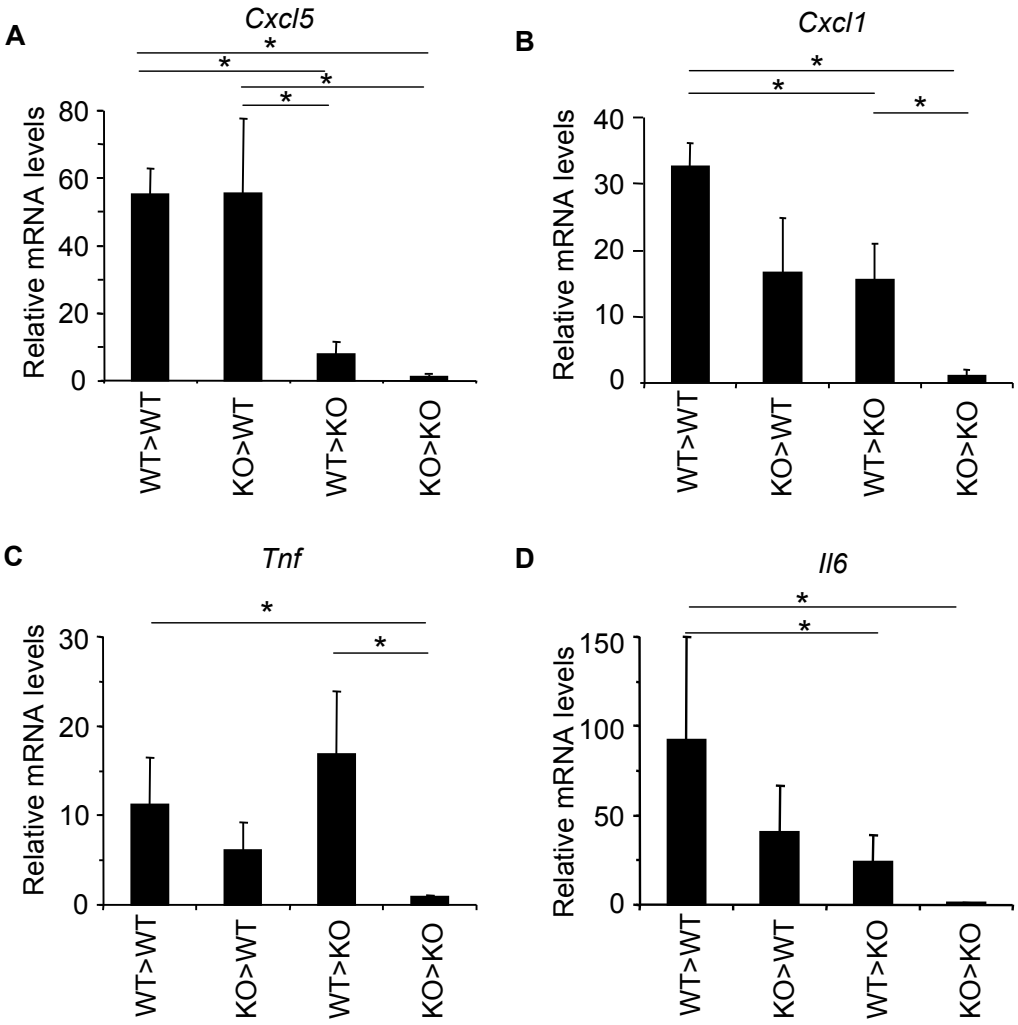

Figure 6 Janot *et al.*