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Original article

Toll-like receptor 4 orchestrates neutrophil recruitment into airways during the first hours of *Bordetella pertussis* infection

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Abstract

Most of the knowledge on the impact of *Bordetella pertussis* lipo-oligosaccharide (LOS) on the infectious process was obtained when the bacteria was established within the host. The aim of the present work was to determine the role of TLR4 at a very early step of the infectious process. To this end we used a transcriptomic approach on *B. pertussis* intranasal infection model in C3H/HeN, a TLR4-competent mouse strain, and C3H/HeJ, a TLR4-deficient mouse strain. The expression of approximately 140 genes was significantly changed 2 h post-infection in the C3H/HeN animals compared to the C3H/HeJ animals, which were essentially non-responders at this early time point. Pathways specific for immunity and defense, chemokine- and cytokine-mediated functions and TLR signaling, were activated upon infection in the TLR4 competent mice either at 2 h or 24 h. Furthermore, we observed that TLR4 signaling is absolutely required to promote the rapid recruitment of neutrophils into the airways. Interestingly, the depletion of those neutrophils impacted on *B. pertussis* lung counts in the first three days, thereby exacerbating the lung infection. In summary, we determined that TLR4 is a central player in initial neutrophil recruitment and orchestration of the very early innate defense against *B. pertussis*.

Keywords: *Bordetella pertussis*; TLR4; LPS; Neutrophils

1. Introduction

Bordetella pertussis causes whooping cough or pertussis, a respiratory disease that is most severe in infants. Before the introduction of childhood vaccination in the 1950s, pertussis was a major cause of infant mortality worldwide. Although the vaccination of children significantly reduced morbidity and mortality, in the 1990s, a resurgence of pertussis was observed in many countries. Today, some countries and some states of the USA have reported a rise in pertussis cases with incidence levels similar to those of the 1940s and 1950s, which was when thorough vaccination was just beginning [1]. Although it is clear that we must use the best cellular and acellular vaccines available, better vaccines are needed [2,3]. The knowledge of the infectious process, in addition to understanding the mechanism by which protection is achieved, is expected to contribute to the development of new vaccines [4].

Although *B. pertussis* is an obligate human pathogen, bacterial colonization of airways in mice has been extensively

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used to provide information about host–pathogen interactions and to obtain knowledge that was essential for the development of anti-pertussis vaccines [5–7]. Using this model, it was determined that several cytokines (i.e., IFNγ and TNF α, among others) and receptors, such as the innate receptor Toll-like receptor 4 (TLR4), were central players in the anti-pertussis response [8,9]. Moreover, TLR4 has been identified as a major genetic factor that modulates anti-pertussis immunity [9–12]. In particular, it was observed that a TLR4-deficient mouse strain is more sensitive to *B. pertussis* infection than a TLR4-competent mouse strain [9–12]. Recently, we reported that the stimulation of TLR4 signaling by nasal co-administration of lipo-oligosaccharide (LOS) with *B. pertussis* promotes a total clearance of bacteria within a few hours, blocking the progression of the infection [13]. To dissect the contribution of TLR4 to the anti-pertussis response during the early phase of the host–pathogen interaction, we describe here a comparative transcriptional analysis of immune-competent and TLR4-deficient mice during infection. We found that TLR4 signaling regulates the expression of genes encoding pro-inflammatory mediators and chemokines specific for immune cells, including neutrophils, in the first 2 h of the infectious process. We also demonstrated that the rapid recruitment of functional neutrophils into airways is essential to the control of *B. pertussis* infection.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The *B. pertussis* strain used in this study was Tohama I (CIP 8132, Collection de l'Institut Pasteur, France). The *B. pertussis* strain was grown on Bordet Gengou (BG) agar medium (Difco, Houston, USA), supplemented with 10% (v/v) defibrinated sheep's blood (BGA plates). After incubation for 72 h at 36 °C in BGA, the bacteria were plated for another 24 h at 36 °C; then, subcultures were suspended in phosphate-buffered saline (PBS; Merck, NJ, USA). The optical density at 650 nm was used to normalize inocula [14]. For each experiment, the inoculum size was determined by bacterial counting using BGA plates.

2.2. Bacterial carboxifluorescein diacetate succinimidyl ester (CFSE) labeling

Up to 10^9 *B. pertussis* CFU were incubated in 1 mL of a 10 μM solution of CFSE (Invitrogen, Carlsbad, CA, USA) for 45 min at 37 °C. The bacterial pellet obtained by centrifugation for 10 min at 10,000 *g* was washed 3 times and diluted as needed for intranasal inoculation.

2.3. Animal infections

Specific pathogen-free C3H/HeN and C3H/HeJ mice were originally obtained from Jackson Laboratories (Bar Harbor, USA) or Harlan (Netherlands). All mice were maintained in the animal care facilities of the Instituto Biológico Argentino (BIOL SAIC) or the Pasteur Institute of Lille (accreditation # A59-350009) in accordance with the institutional guidelines. Mice were lightly sedated with isoflurane and infected intranasally (i.n.) with the appropriate number of CFUs of *B. pertussis* in 20 μl of PBS. Infections were accomplished by pipetting the inoculum onto the nostrils. Animals were euthanized at the indicated times, and the bacterial colonization of the lungs was quantified as previously described [14].

2.4. Tissue processing, RNA isolation, reverse transcription and quantitative real-time PCR

C3H/HeN and C3H/HeJ mice were intranasally inoculated with 20 μL of PBS or different bacterial loads of *B. pertussis* (5×10^3, 5×10^5, 5×10^7 CFU). The mice were euthanized by cervical dislocation at 2 h post-infection. The inferior lobe of the right lung was immediately processed for total RNA isolation using the Nucloespin RNAII system (GE, USA) following the manufacturer's instructions. RT-qPCR was performed as described [13,14] using 100 ng of total RNA and MMLV-RT (Promega). The resulting cDNA was amplified in triplicate using the SYBRR Green PCR assay (Bio Rad Laboratories, Hercules, CA, USA), and the products were detected on an ICycler (BioRad). The PCR samples were incubated for 2 min at 50 °C and for 10 min at 95 °C, followed by 40 amplification cycles with a 1 min annealing/extension step at 60 °C and 15 s denaturation at 95 °C. The expression of β-actin was used to normalize the data. The specificity of the PCR reaction was confirmed by melting curves. Relative mRNA levels were determined by comparing the normalized PCR cycle threshold (Ct) between cDNA samples of the gene of interest. The specific primers for *il6* were: Fwd: 5′GTT CTC TGG GAA ATC GTG GAA A 3′; Rev: 5′AAG TGC ATCATCG TTG TTC ATA CA 3′. The specific primers for *tnf α* were: Fwd: 5′CAT CTT CTC AAA ATT CGA GTG ACA A 3′; Rev: 5′CCT CCA CTT GGT TTG CT 3′, and the specific primers for *cxcl1* were: Fwd: 5′CTT GGT TCA GAA AAT TGT CCA AAA 3′; Rev: 5′CAG GTG CCAT CAG AGC AGT CT 3′. The animal experiments were performed at least in triplicate.

2.5. Microarray analysis

Four mice were processed for each condition. For each mouse strain, uninfected control mice were used as the reference control. Total RNA was extracted from lungs using NucleoSpin® RNA L (Macherey–Nagel, Duren, Germany) and was concentrated in a YM30 Microcon centrifugal filter device (Millipore, Billerica, MA, USA) by centrifugation at 14,000 *g* for 60 min at 4 °C. Poly(A) mRNA from 24 μg of total RNA was converted into digoxigenin-labeled cDNA for hybridization to Mouse Genome Survey Microarrays V2 (Applied Biosystems, Carlsbad, CA, USA). Signals were detected using the Chemiluminescent RT Detection Kit, and the microarrays were scanned with an Applied Biosystems ABI1700 instrument as described previously [15]. The raw microarray data were quality controlled using previously defined procedures [16,17]. Data from different biological conditions were compared in an "everyone against everyone" scheme, and log 2 quotients ("log *Q*") were then determined as the mean of the weighted individual log *Q* values [15]. For these inter-

assay comparisons, the NeONORM method was used [18]. *p*-values were determined using a *t*-test and a pFDR correction. Multiple probes for a single gene and cross-reactivity of a single probe to several genes, as well as the resolution of probe-ID annotations, was accomplished according to the standards defined previously [19]. Heat-maps were created according to standard methods. To underline the single-color chemiluminescence detection character of the AB1700 system, we used a blank (signal = 0) to bright green (signal >10) gradient to represent the signal intensities. Combining GO, Kegg and PANTHER annotations, we assigned all the probes on the microarrays to pathways or biological processes. We then calculated the relative representation of the detected probes as significantly regulated. *p*-values for over- and under-representation of pathways were calculated using a binominal distribution with a Bonferroni correction for multiple testing. The complete dataset is available from the MACE database at http://mace.ihes.fr under accession no.: 2400552118.

2.6. TaqMan low density array (TLDA)

Total RNA was isolated using the Nucleospin RNAII system (Macherey–Nagel) as described. The synthesis of cDNA was performed with 100 ng of RNA for each sample using a High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Gene expression was analyzed using a custom designed TLDA in a 7900 Real-Time PCR System (Applied Biosystem) following the conditions stated by the manufacturer. Analysis was carried out using the Real-Time StatMiner software from Integromics (Philadelphia, USA). Relative mRNA levels ($2^{-\Delta\Delta Ct}$) were determined as previously described [15] by comparing: (i) the PCR cycle thresholds (Ct) for the gene of interest and the Cts of 5 normalizing genes (*Actb*, *18S*, *Dpp*, *Polr2j*, *Ube3b*) to calculate the ΔCt; and (ii) the ΔCt values for treated and control groups (ΔΔCt). In all experiments, the Ct upper limit was fixed to 33 cycles. The list of genes analyzed is given in Supplementary Table 1.

2.7. Bronchoalveolar lavage (BAL) and lung cell analyses

Infected and control mice were euthanized by intraperitoneal pentobarbital injection and the thoracic cavity was dissected. After severing the descending aorta, the blood in the lungs was cleared by perfusing PBS at room temperature through the heart's right lobe until the lungs became pale. To perform the BAL, the trachea was partially cut and 1 mL of sterile PBS containing 0.1% BSA was flushed into the lungs and then withdrawn. This procedure was repeated 3 times. The resultant cells were then washed, resuspended and counted in a Neubauer chamber. To obtain cell suspensions from whole lungs, the lungs were collected in 2 mL of digestion medium (RPMI without mercaptoethanol, 5% FCS, 1 mg/mL collagenase A, and 40 μg/mL DNAse I), incubated for 30 min at 37 °C, mechanically disrupted and further incubated for 5 min at 37 °C. The recovered cells were washed with 5 mL of cold wash medium (PBS containing 2% FCS and 5 mM EDTA) and filtered through a 40 μm cell strainer (Becton Dickinson, USA). To remove red blood cells, the BAL fluid was treated with Red Cell Lysis Buffer (BD) for 3 min at room temperature. Finally, the cells were counted in a Neubauer chamber to determine the absolute number.

2.8. Immunomagnetic separation of bronchoalveolar, lung and blood GR1$^+$ cells

Cell enrichment was performed using beads specific for mouse Ly-6G and Ly-6C (Gr1) (BD Imag™, BD Biosciences). Briefly, 50 μl of beads were added for every 10^7 cells (input cells) from BAL or nucleated cells from blood, previously treated with red cell lysis buffer (BD Biosciences) and separated in a Ficoll gradient (BD Biosciences). The cells and beads were mixed thoroughly and incubated at 6–12 °C for 30 min. The labeled cell suspension was brought up to $1-8 \times 10^7$ cells/mL in PBS containing 2% FCS and 5 mM EDTA and placed within the magnetic field. The Gr1-positive and Gr1-negative fraction were then isolated. The washing procedure was repeated five times and cell enrichment was confirmed by FACS. Depending on the condition studied, the purity of the fraction ranged from 90 to 99%. Separated cell fractions, as well as flow-through and input samples from infected and control mice, were used for qPCR analysis as described above.

2.9. Analysis of leukocytes of the bronchoalveolar space

Cells from the BAL were stained with fluorescent antibodies for 1 h at 4 °C, followed by flow cytometry analysis using a FACSCalibur from Becton Dickinson. The FITC-, PE-, APC-, and PerCP-conjugated monoclonal specific antibodies for CD11c (clone N418, hamster IgG: eBioscience, San Diego, CA, USA), Gr1 (clone RB6-8C5, Rat IgG2b: eBioscience), CD11b (clone M1/70.15, Rat IgG2b: Caltag Laboratories, CA, USA), Ly-6G (clone 1A8, Rat IgG2a: BD Pharmingen) and Ly6C (AL-21, Rat IgM: BD Pharmingen) were used to label the cells. In the cases of the CFSE-labeling of bacterial cells, the acquisition parameters for the green channel were adjusted and specific antibody-conjugated fluorophores were selected to obtain an appropriate separation of cell populations in the different acquisition channels.

2.10. Neutrophil depletion

Neutrophils were depleted by an intraperitoneal (i.p.) administration of a purified rat monoclonal antibody (RB6-8C5, 2.0 mg/mL) specific for Ly-6G/Ly-6C (Gr-1) or an isotype control antibody HB152. The dosing schedule consisted of the administration of 400 μg of antibody the day before the intranasal inoculation with *B. pertussis*, followed by 200 μg of antibody every other day until mouse necropsy. The efficacy of the treatment with anti-Gr1 antibody was controlled by its capacity to deplete 95% of the neutrophils in the lung of flagellin- or LPS-stimulated animals. In the infection experiments,

efficacy of the depletion was confirmed by a reduction of at least 95% in the neutrophil population in either blood or spleen.

3. Results

3.1. TLR4-dependent signature in the early stages of B. pertussis infection

To determine the role of TLR4 at a very early step of the infectious process, we measured the expression of pro-inflammatory genes in two mouse strains at 2 h post-bacterial intranasal infection: C3H/HeN, a TLR4-competent mouse strain, and C3H/HeJ, a TLR4-deficient mouse strain. Knowing that both mouse strains have different susceptibilities to *B. pertussis* infection, we evaluated the induced immune response using different doses of bacteria (5×10^3–5×10^7 CFU). The results included in Table 1 show that in the C3H/HeN mice, the highest dose strongly induced the transcription of *il6*, *tnf α* and *cxcl1* genes as soon as 2 h post-infection. In contrast, in the C3H/HeJ strain, only a marginal increase of *tnf α* expression and a trend in *cxcl1* upregulation was detected at the highest dose.

When bacterial lung colonization was analyzed in mice infected with a high dose of *B. pertussis*, we observed differences in the bacterial infection kinetics. It is important to note that both C3H/HeN and C3H/HeJ mice were equally infected because similar numbers of bacteria were detected in lungs 2 h post-infection. In agreement with previous studies [20], at 24 h post-infection, differences in bacterial load were detected in both mouse strains. We observed that $1.0 \pm 0.1 \times 10^7$ CFUs were recovered from the lungs of C3H/HeJ infected mice, and $6.8 \pm 0.2 \times 10^6$ CFUs were recovered from C3H/HeN infected mice ($p \leq 0.01$). These differences were sustained at 48 h post-infection ($8.9 \pm 0.2 \times 10^6$ CFUs C3H/HeJ and $1.6 \pm 0.2 \times 10^6$ CFUs C3H/HeN; $p \leq 0.01$) and later time points (data not shown), following a kinetics comparable with previous reports [9,20].

To further analyze the contribution of TLR4 signaling during the early steps of the infection, comparative transcriptomic analyses at 2 h and 24 h post-infection were performed at the highest infective dose in both mouse strains (Fig. 1). As a control, PBS-treated uninfected mice were used. Non-significant differences in the gene expression pattern between the control mice of both strains were observed (data not shown). However, at 2 h after *B. pertussis* infection, the expression of 142 genes was significantly different in the C3H/HeN mice compared to the PBS-treated controls. The majority of these genes were found to be transcriptionally up-regulated. Remarkably, under the same conditions, only 12 genes were differentially regulated in C3H/HeJ animals; most of these genes were also induced in the TLR4 competent strain (Fig. 1A).

Compared to uninfected animals, the transcription of 687 and 918 genes was differentially regulated at 24 h post-infection in C3H/HeN and C3H/HeJ mouse strains, respectively. In both strains, two-thirds of the total genes were up-regulated and one-third were down-regulated (Fig. 1A). C3H/HeN and C3H/HeJ mouse strains had 462 genes that were differentially modulated, corresponding to 50% of the genes in the C3H/HeJ strain and 65% in the C3H/HeN strain (Fig. 1B). The coincidence between both mouse strains was 79% if the top-100 up-regulated genes were considered (Fig. 1B) and 98% if the top-50 up-regulated genes were considered (data not shown). Interestingly, these results indicated that a dominant transcriptional response characterized by pathways, such as immunity and defense, interferon-mediated immunity, macrophage-mediated immunity, and proteolysis, is shared by both mouse strains at 24 h post-infection (Table 2). In contrast, at 2 h post-infection, the C3H/HeN mouse strain but not the C3H/HeJ mouse strain responded to bacterial infection. For the C3H/HeN mouse strain, 40% of the genes induced at 2 h were still up-regulated at 24 h post-infection (Fig. 1B). Furthermore, for this mouse strain, only 20% of the 100 genes up-regulated to the highest level were similar at 2 h and 24 h, which is characteristic of a TLR-mediated response that is characterized by fast- but transient-induction of gene expression.

Gene ontology analysis allowed us to determine that the pathways specific for immunity and defense, chemokine- and cytokine-mediated functions and TLR signaling, were activated upon infection in the TLR4 competent mice either at 2 h or 24 h (Table 2 and Supplementary Table 2), whereas in the TLR4 mutant mouse strain, almost no response was detected at 2 h post-infection. Neutrophil-attracting chemokines (*cxcl1*, *cxcl2* and *cxcl5*), monocyte/macrophage-attracting chemokines (*ccl2*, *ccl3*, *ccl4*, *ccl7*) and T-cell-attracting chemokines (*cxcl10*, *ccl17*, *ccl22*) were among the genes induced to the greatest amount in C3H/HeN mice (Fig. 2A). Furthermore, genes coding for the hallmark cytokines of innate immune response activation, such as *tnf*, *il1b*, and *il6*, were highly induced. The induction of the expression of various antimicrobial molecules was also detected (encoded by *ptx3*, *saa3*, and *lcn2*), as well as the overexpression of neutrophil-activating signals, such as G-CSF encoded by *csf3* (Supplementary Table 2).

To better characterize the kinetics of gene transcription within the first hours of infection (2 h, 6 h and 24 h post-infection) and to validate the microarray data, we performed qPCR on representative genes of the response (Fig. 2B). Analysis of lung tissues at those time points highlighted the fact that pro-inflammatory gene expression was more rapidly and strongly induced in the TLR4-competent than in the TLR4-deficient mouse strain. For example, the gene encoding the proinflammatory cytokine *il1b* was up-regulated 9.1-fold at 2 h in the TLR4 competent strain

Table 1
Gene expression changes in mouse lung induced by infection with *B. pertussis* at different bacterial loads. C3H/HeN and C3H/HeJ mice ($n = 4$) were intranasally inoculated with PBS or *B. pertussis* (5×10^3, 5×10^5 or 5×10^7 CFU). Transcript abundance in the lung was assayed by qPCR and normalized to b actin level. Values indicate the fold change ± SD relative to uninfected controls. a $p \leq 0.01$ vs. control and b $p \leq 0.01$ vs. counterpart mouse strain.

Gen	Mice	Treatment/dose of bacteria			
		PBS	5×10^3	5×10^5	5×10^7
tnf a	C3H/HeN	1.1 ± 0.6	0.4 ± 0.2	1.0 ± 0.4	11.5 ± 4.5[a,b]
	C3H/HeJ	1.1 ± 0.4	1.0 ± 0.5	1.0 ± 0.1	3.5 ± 1.6
il6	C3H/HeN	1.5 ± 1.6	0.3 ± 0.2	0.7 ± 0.4	25.9 ± 2.4[a,b]
	C3H/HeJ	1.1 ± 0.9	0.4 ± 0.5	1.1 ± 0.3	2.0 ± 1.7
*cxcl*1	C3H/HeN	1.1 ± 0.1	0.7 ± 0.8	1.6 ± 1.0	17.6 ± 6.9[a,b]
	C3H/HeJ	1.1 ± 0.4	1.1 ± 1.1	1.3 ± 0.2	6.2 ± 4.1

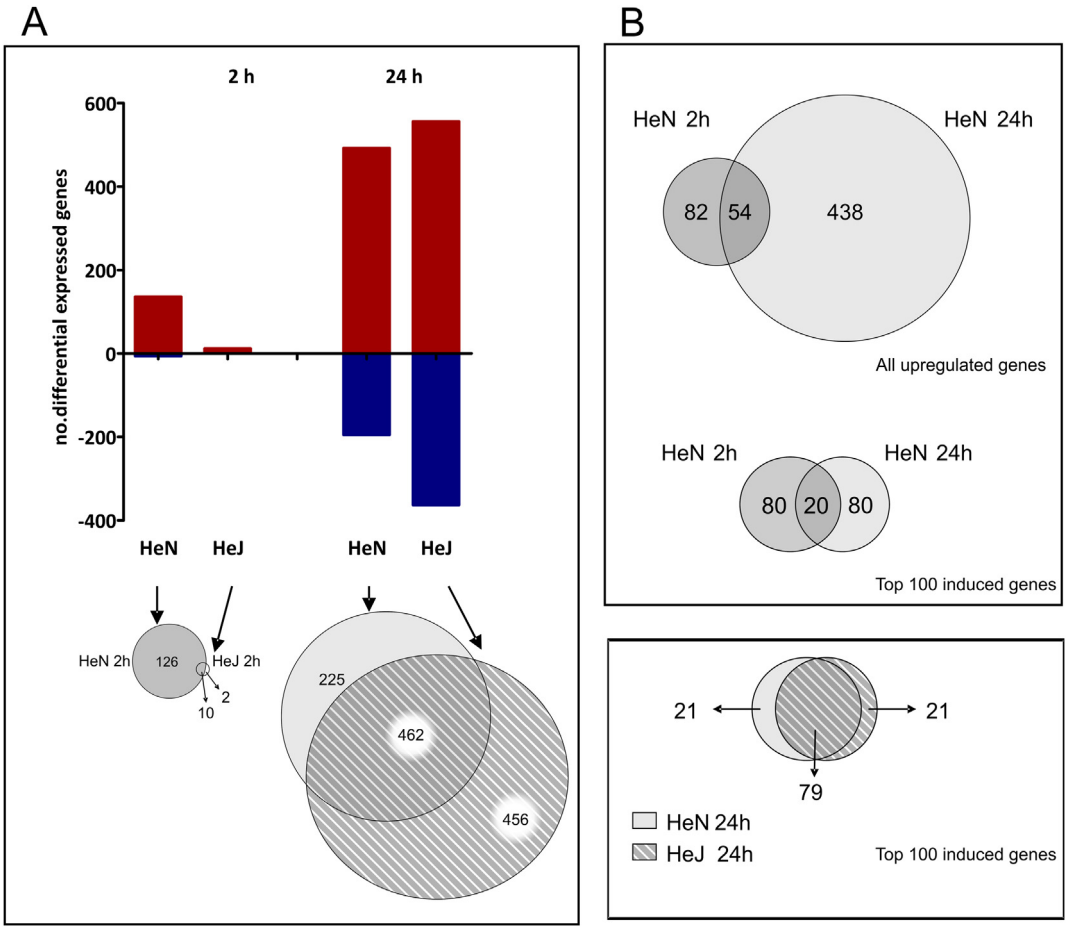


Fig. 1. TLR4 is critical for a robust and immediate lung innate response. The transcriptional response to *B. pertussis* infection was measured using Mouse Genome Survey V2 microarrays in C3H/HeN and C3H/HeJ at 2 h and 24 h ($n = 4$ per condition). A. Upper panel: the number of genes differentially modulated during infection. The up-regulated genes are depicted in red and the down-regulated genes are shown in blue. Lower panel: the diagram shows the number of shared genes or specific genes differentially modulated for each mouse strain at both time points. B. Upper diagram: the number of up-regulated genes, shared or specific, for different time points in TLR-4 competent mice. A detail on the overlapping of the 100 most up-regulated genes in each condition is also shown. Lower diagram: the number of up-regulated genes, shared or specific, for each mouse strain at 24 h post-infection. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

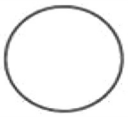
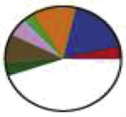
when compared to control mice, whereas it was induced 2.1-fold in the TLR4-deficient strain. Different groups of genes showed different kinetics of response. Some genes, such as *tnfa*, *il6* or *ptx3*, have their maximal induction at very early time points, and their expression drops to almost similar levels between both mouse strains at 24 h. On the other hand, expression of *cxcl2*, *cxcl10* or *il1b* peaked at 6 h, whereas *ifng* transcription increased up to 24 h post-infection. For most genes, the transcriptional activation was higher in the TLR4-competent mice than the deficient counterpart, and the differences were more evident at 2 h or 6 h post-infection, with a tendency to show a similar magnitude at 24 h. In accordance with the microarray analysis, TLR4 signaling promoted a fast and strong induction of the innate response.

3.2. *Early protection against* B. pertussis *infection is associated with TLR4-dependent neutrophil recruitment*

Because TLR4 regulates the early expression of genes encoding for chemo-attractants, we next analyzed leukocyte recruitment into the lung and airways. Cells from broncho-alveolar lavages (BAL) were sampled at different times after *B. pertussis* infection and analyzed by flow cytometry (Fig. 3). In uninfected animals, alveolar macrophages identified as $CD11c^{+}CD11b^{-}Ly6G^{-}Gr1^{-}$ cells were the predominant cells in both mouse strains (Fig. 3A). After infection, non-significant changes in the absolute number of alveolar macrophages were observed (Fig. 3C).

In contrast, a population characterized by the surface markers $CD11c^{-}$ $CD11b^{+}$, which are absent in uninfected lungs, was massively recruited into the broncho-alveolar compartment of the lungs of C3H/HeN mice (Fig. 3A). This population, expressing the neutrophil-specific markers Gr1 and Ly6G (Fig. 3B), was detectable at 2 h with approximately 10^3 neutrophils/BAL and continued to rise to $>10^6$ neutrophils/BAL at 24 h post-infection (Fig. 3C). In C3H/HeJ mice, the kinetics of neutrophil recruitment were delayed and the magnitude of infiltration was about 10-fold less than in TLR4 competent mice at the different time-points (Fig. 3C). Altogether, the gene

Table 2
Functional analysis of differentially regulated genes at 2 and 24 h post-infection in TLR4 competent and deficient mouse strains. The analysis of pathways involved in the host response was performed using GO, KEGG and PANTHER annotations. *p*-values were calculated based on the number of genes in the pathway that were differentially regulated compared with the number of genes that would be retrieved by random behavior.

	2h		24h		24h
Ontology	C3H/HeJ	C3H/HeN	C3H/HeJ	C3H/HeN	HeJ & HeN
					
Immunity and Defense	1.00 E+00	2.14 E-16	2.40 E-53	7.86 E-66	1.06 E-49
Interferon-mediated Immun.	1.00 E+00	7.71 E-01	2.00 E-29	5.89 E-38	4.68 E-33
Macrophage-med. Immun.	1.00 E+00	2.76 E-04	4.74 E-27	4.56 E-20	3.02 E-18
Proteolysis	1.00 E+00	4.86 E-01	1.38 E-14	1.29 E-16	1.03 E-13
Cytokine & Chemok. Immun.	1.00 E+00	5.58 E-12	2.44 E-13	1.01 E-14	4.65 E-13
Cytokine / Chemok. Immun.	1.00 E+00	1.36 E-10	8.29 E-13	1.75 E-13	1.53 E-12
Ligand-mediated Signaling	1.00 E+00	4.23 E-10	4.61 E-08	2.77 E-08	1.59 E-09
Natural Killer Cell Immun.	1.00 E+00	1.00 E-00	2.01 E-07	4.99 E-09	2.64 E-07
Signal Transduction	1.00 E+00	1.60 E-08	3.72 E-04	6.86 E-04	2.09 E-03
Cell communication	1.00 E+00	2.87 E-08	5.89 E-07	1.43 E-04	8.06 E-05
Cell prolif. & differentiation	1.00 E+00	4.57 E-07	1.82 E-02	8.48 E-04	4.15 E-03

expression data correlated with neutrophil infiltration, demonstrating that the TLR4-competent mice are able to recruit neutrophils faster and in higher numbers into airways in the first 24 h of *B. pertussis* infection.

Because the neutrophil infiltration could influence the disease outcome, we analyzed the impact of the depletion of $Gr1^+$ cells on the onset of *B. pertussis* infection in C3H/HeN mice. We observed that this cell depletion caused a significant increase in bacterial number in the lungs at 24 h post-infection ($p \leq 0.01$) (Fig. 3D). These differences were sustained at 48 h post-infection and the bacterial load was even higher at 72 h post-infection ($p \leq 0.01$) (Fig. 3D). This result indicates that $Gr1^+$ cells contribute significantly to the control of the infection. Taken together, our results suggest that TLR4 signaling plays a key role by amplifying early neutrophil recruitment into airways, thereby promoting an increased clearance of bacteria at an early time and promoting a faster termination of colonization.

To assess the functional capacities of neutrophils recruited to BAL at the initial steps of the infection in TLR4 competent and deficient mice, additional experiments were performed. We determined that airway neutrophils were strongly associated with CFSE-labeled *B. pertussis* in C3H/HeN mouse strain (Fig. 4A, B). A similar association between airway neutrophils and *B. pertussis* was observed in C3H/HeJ mouse strains (Fig. 4B). Using cells isolated from lung, we observed that the bacterial 16S rRNA levels were strongly increased in the $Gr1^+$ neutrophil population at 24 h post-infection in both mouse strains (Fig. 4C). These results indicated that neutrophils have a TLR4-independent capacity to interact with *B. pertussis.*

To define the impact of TLR4 on neutrophil activation in both mouse strains, we determined the transcriptional activity of the cells isolated from BAL of infected mice. As reference we used neutrophils isolated from blood of non-infected mice. The transcriptional activity of pro-inflammatory genes such as *cxcl10*, *il1b*, *cxcl2*, *lcn2* and *csf3* is indicative of the activation status of the neutrophils. These markers were increased in $GR1^+$ cells purified from the BAL of infected mice 24 h post-infection, compared to isolated $GR1^+$ cells from peripheral blood (Fig. 4D). The $GR1^+$ cells from peripheral blood from uninfected animals of both mouse strains presented comparable levels of expression of these genes. Moreover, blood neutrophils from infected animals showed gene expression levels similar to uninfected mice, indicating that neutrophil transcriptional activation is taking place exclusively at the site of the infection. On the other hand, alveolar neutrophils isolated from BAL upon infection showed a strong transcriptional activity of these markers compared to blood-derived neutrophils, either in C3H/HeN and C3H/HeJ strains. These results indicate that neutrophil activation during *B. pertussis* infection is independent of TLR4.

Because neutrophils appeared to be functionally similar despite the TLR4 signaling capacity, we conclude that the TLR4-dependent kinetics and the magnitude of cell recruitment are the main driving forces of the protective effects.

4. Discussion

Using a transcriptomic approach focused on the very early host response to *B. pertussis* infection, we showed that TLR4 is essential to trigger a rapid host response. Two mouse strains, C3H/HeN and C3H/HeJ, that are TLR4-proficient and TLR4-deficient respectively, were used in the gene profiling experiments reported in this work. The expression of approximately 140 genes was significantly changed 2 h post-infection in the C3H/HeN animals compared to the C3H/HeJ animals, which

A

Gen Symbol	C3H/HeN		C3H/HeJ		Recruitment
	2 h	24 h	2 h	24 h	
cxcl1	4,9	3,1	2,9	3,6	Neutrophills
cxcl2	5,1	ns	ns	ns	Neutrophills
cxcl5	3,9	3,9	ns	3,5	Neutrophills
cxcl9	ns	6,1	ns	5,5	Th1,NK
cxcl10	3,9	5,7	ns	5,5	Th1,NK
cxcl11	2,5	6.8	ns	6,5	Activated T cells
cxcl12	ns	ns	ns	1,9	T Cells
cxcl13	ns	3,1	ns	3,8	B Cells
cxcl16	ns	1,2	ns	1,2	T Cells & NKT
ccl2	2,5	2,9	ns	3	Monocyte, Activated T Cells, NK
ccl3	3	2,9	ns	3,1	Monocyte, T Cells, DC
ccl4	3,9	3,5	ns	3,1	Monocyte, T Cells, DC
ccl7	3,4	4,1	ns	4,2	Monocyte, Activated T Cells, NK, Eos
ccl8	ns	2,6	ns	2,5	Monocyte, Activated T Cells, NK, Eos
ccl9	ns	1,5	ns	2	Several
ccl17	2,1	ns	ns	ns	Th2
ccl19	ns	1,9	ns	2	T & B Cells
ccl20	2,9	ns	ns	ns	Inmature DC, Activated B & T Cells
ccl22	2,1	ns	ns	**ns**	Th2

B

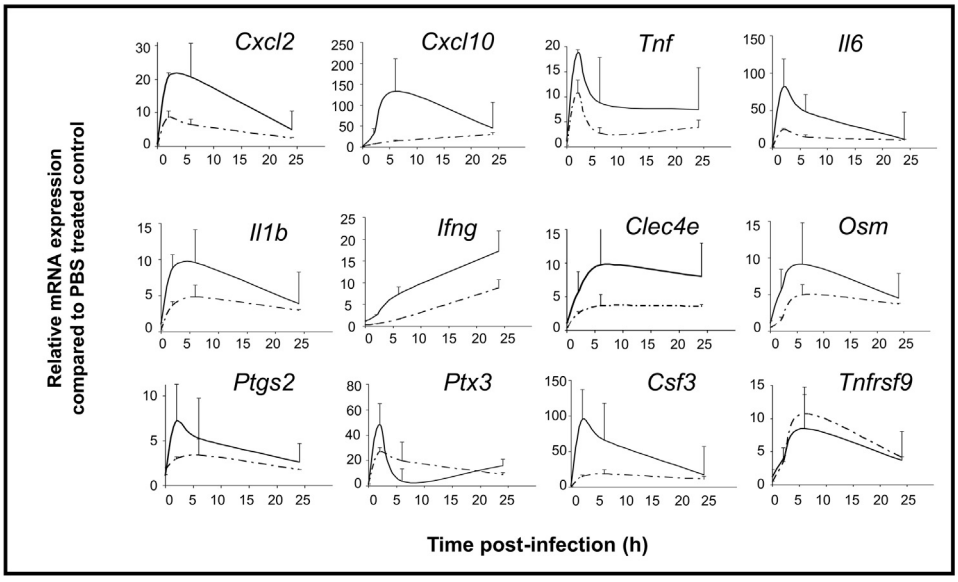


Fig. 2. Lung proinflammatory gene expression depends on TLR4 functionality, especially at early time points. A. Chemokine lung expression is TLR4-dependent during *B. pertussis* infection. Log *Q* indicates the log 2 of the ratio of the expression of infected vs. control (PBS-treated) mice for each gene. B. Kinetics of gene expression depends on TLR4 functionality. C3H/HeN (solid line) and C3H/HeJ (dashed line) mice ($n = 3$) were infected with 5×10^7 CFU of *B. pertussis*. The expression level of a set of innate activation markers was determined by the RT-qPCR on total lung RNA at 2, 6 and 24 h post-infection. The mRNA levels are expressed relative to the PBS group. The results are given as the mean ± SD. Similar results were obtained in 2 independent experiments.

were essentially non-responders at this early time point (Fig. 1A). These results indicate that despite a high infective dose of microorganisms, which display several PAMPs and host signaling subversive toxins, TLR4-dependent signaling is clearly the main component of the initial anti-pertussis response. With the progress of *B. pertussis* infection, the transcriptional response detected in both mouse strains became more comparable, indicating a stereotypical immune response biased to antimicrobial defense and inflammation (Table 2, Fig. 2), probably contributed by other PAMP/DAMP sensors in TLR4-deficient mice [21,22]. Therefore, the early response orchestrated in TLR4-competent mice seems to affect *B. pertussis* kinetics colonization. In fact, in agreement with previous reports by Higgins et al., 2003, lower bacterial loads were detected in TLR4-competent mice after 24 h post-infection, in comparison with TLR4-deficient infected mice [9]. Their analysis showed that the initial immune response was faster and stronger in the TLR4-competent mice and was delayed in the TLR4-deficient mouse strain, however they focused their attention in the later times of infection. Several days after infection, the authors found a massive recruitment of neutrophils and cytokine expression in the TLR4-deficient mouse strain, which was consistent with lower IL10 production and a bacterial burden that was at least three orders of

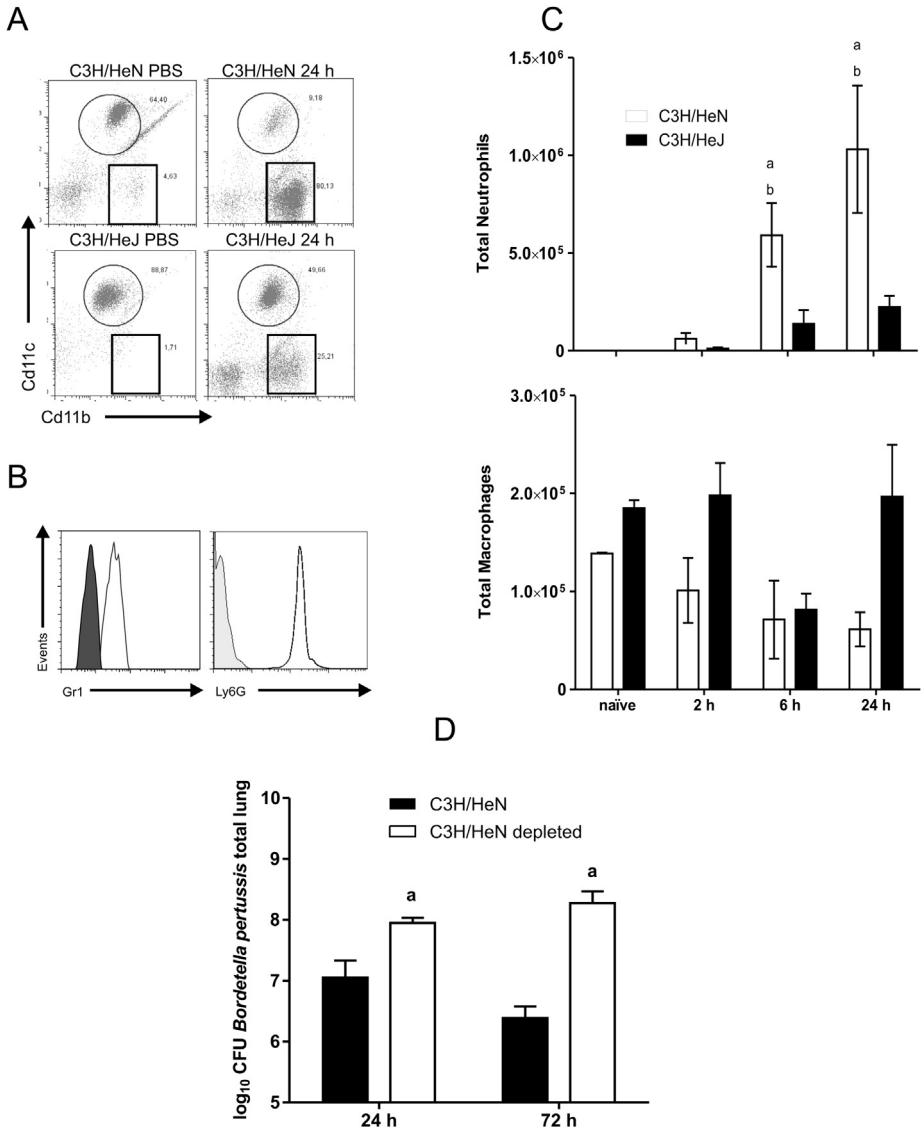


Fig. 3. Leukocyte recruitment into the airways after *B. pertussis* infection is TLR4-dependent. C3H/HeN and C3H/HeJ mice ($n = 5$) were infected intranasally with 5×10^7 CFU of *B. pertussis*. Cellular populations in airways were sampled by broncho-alveolar lavage (BAL) and analyzed by flow cytometry at the indicated time points. A. BAL dot-plots representative of infected and control mice of both strains. Alveolar macrophages are depicted in this representation as CD11b−CD11c$^+$ and neutrophils as CD11b$^+$ CD11c$^-$ cells. B. Expression of Gr1 and Ly6G markers on CD11b$^+$ CD11c$^-$ cells. Isotype control staining is shown in gray. C. Quantitative analysis of BAL leukocytes from naïve and infected mice. The results representative of 2 experiments are given as the mean ± SEM. a indicates $p < 0.05$ vs. control, b indicates $p < 0.05$ vs. counterpart mouse strain. D. Gr1$^+$ cells contribute to the early control of infection. C3H/HeN mice ($n = 4$) were infected intranasally with 5×10^7 *B. pertussis* and treated i.p. with depleting anti-GR1 or control antibody. At the indicated times, lungs were sampled and bacterial load determined. (■ mice treated with isotype control, □ mice treated with depleting anti-GR1 antibody.) a indicates $p < 0.05$. Similar results were obtained in three independent experiments.

magnitude higher than the levels observed in the TLR4-competent mice [9].

Our transcriptional analyses at 24 h post-infection are consistent with those reported by Banus and colleagues using *B. pertussis* Tohama phase I [23,24] because 66% of the differentially regulated genes are shared between our results and theirs at 24 h post-infection. Although there are differences in the experimental conditions and data analyses (i.e., mice strains, microarray platforms, among others), the Pearson correlation coefficient based on the fold increase of transcription was $r = 0.74$. Similar values were obtained when compared to other sets of microarray data that were generated under different conditions and were considered highly significant [25,26]. In a recent report, Connelly and colleagues [27] described the functional genomic host response in the first days of infection. Consistent with the results reported here, they found a robust pro-inflammatory response elicited by the wild-type *B. pertussis* infection. The core transcriptional response described by those authors included the same genes that were identified as highly up-regulated in this study.

The inclusion of the 2 h time point in our analysis allowed us to determine that TLR4 is the main sensor of *B. pertussis* at

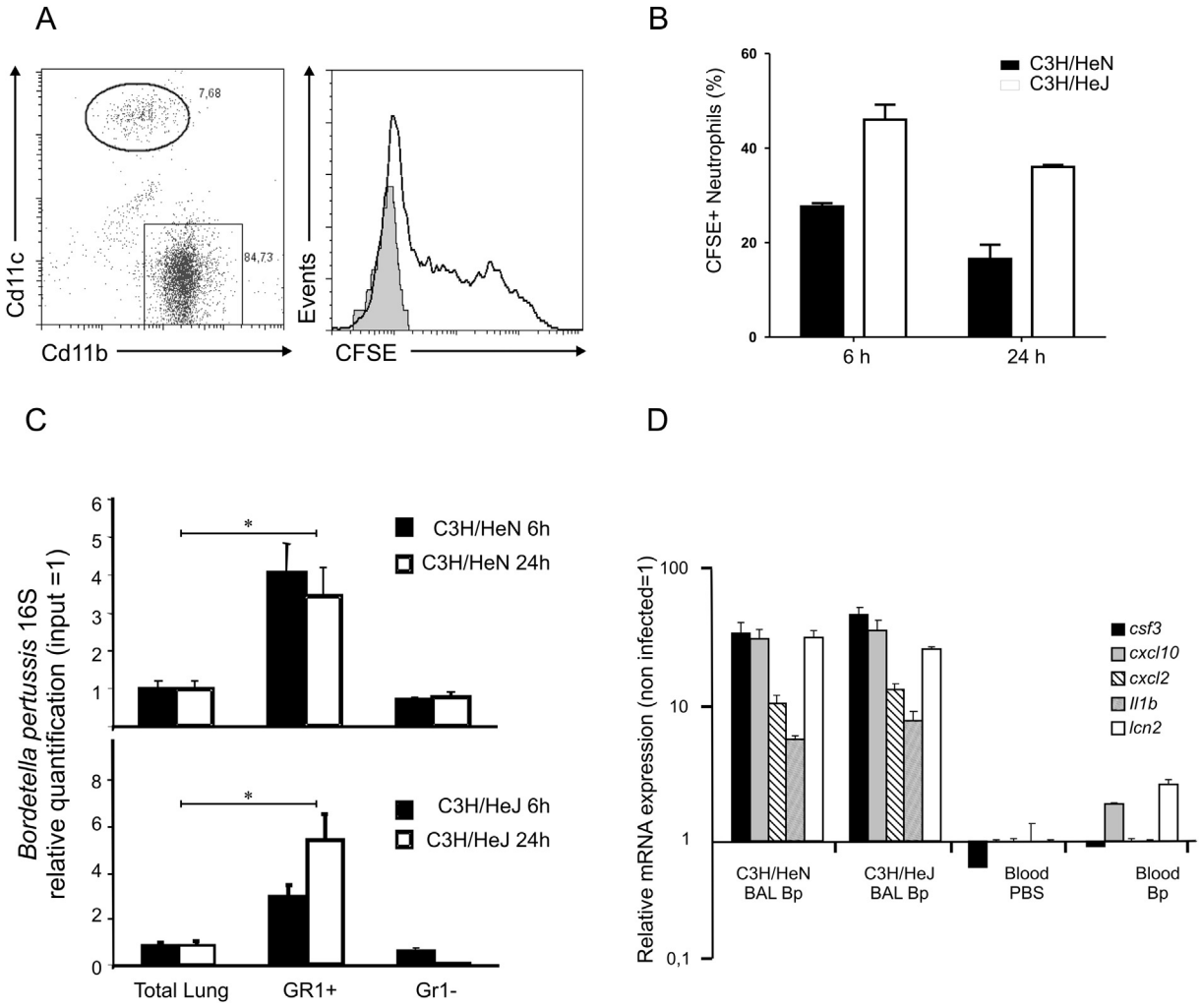


Fig. 4. Neutrophil activation is not impaired in the TLR4-deficient strain. C3H/HeN and C3H/HeJ mice ($n = 3$) were infected intranasally with 5×10^7 CFU of *B. pertussis*. BAL, lung and/or blood were sampled for cellular and molecular analysis at indicated times. A. Infection was performed with CFSE-labeled *B. pertussis* and the association of bacteria with different BAL populations was determined by flow cytometry. $CD11b^+$ $CD11c^-$ cells incorporated the CFSE label (black line) compared to control animals infected with unlabeled *B. pertussis* (gray). The results shown are representative from 2 experiments. B. Percentage of CFSE + neutrophils ($CD11c^-$, $CD11b^+$) in BAL at different times post-infection. Similar results were obtained in two independent experiments. C. Bacteria-associated gene expression in the $Gr1^+$ population. *B. pertussis* 16S gene expression was assessed by qPCR in the lung cell fractions or total lung from infected mice. The 16S RNA level is expressed relative to whole lung. *Indicates $p < 0.05$ vs. whole lung. Similar results were obtained in 2 independent experiments. D. Comparative gene expression of the $Gr1^+$ population obtained from BAL of C3H/HeN and C3H/HeJ mice ($n = 3$) or blood assessed 24 h post-infection. The mRNA levels are relative to blood $GR1^+$ from uninfected controls.

the very beginning of the infection. The early transcriptional response induced by *B. pertussis* in TLR4 competent mice is similar to the lung response to LPS [28] or more broadly to TLR-dependent activation [29,30]. In particular, the transcription of genes encoding for various chemokines specific for neutrophils (*cxcl1*, *cxcl2* and *cxcl5*) were found to be up-regulated (Fig. 2). This transcriptional activation was associated with an increased influx of neutrophils into the airways of TLR4-competent mice very early upon infection. Moreover, we observed that neutrophils are critical to contain the infection (Fig. 3). Vandebriel and collaborators also showed that at 24 h post-infection, *B. pertussis* co-localized with neutrophils [31]. Andreasen and Carbonetti reported that neutrophils participate in the clearance of *B. pertussis* in animals immunized with pertussis vaccine [32]. Our work showed that the early recruitment of neutrophils is dependent, at least in part, on TLR4 signaling. Our study also revealed that TLR4 stimulates the production of factors that are potent activators of neutrophils, such as *il1b*, *il6*, *csf* and *tnfa* (Fig. 2 and Supplementary Table 2) [33]. Furthermore, molecules with antimicrobial capacity and/or enhancers of complement activation and phagocytosis, such as long pentraxin 3 (*ptx3*), lipocalin 2 (*lcn2*) and serum amyloid protein 3 (*saa3*), were also highly expressed in TLR4 competent mice. SAA3 is produced in extra-hepatic tissues under pro-inflammatory conditions and presents pleiotropic activities, enhancing the immune response and activating neutrophils by targeting the formyl peptide receptor [34,35]. In agreement with Connelly and coworkers [27], we observed that *saa3* is one of the most abundant transcripts at 2 or 24 h post-infection in both mice

strains, which may also explain neutrophil activation in TLR4-deficient mice (Supplementary Table 2). Beyond the significant changes in neutrophil recruitment in the first hours of infection changes in the monocyte/macrophage populations in the BAL are expectable after 24 h of infection, according to chemokines upregulated in both strains.

Interestingly, we recently observed that TLR4 signaling induced by LPS stimulated the production of TNF, IL-6, and CXCL2 in the respiratory tract and elicited a protective response against a high challenge dose of *B. pertussis* [13]. Although all these results point to the LPS as the main responsible for the immune response detected, we do not rule out the potential contribution of pertussis toxin since it has been reported that it is also able to bind and activate TLR4 pathway [36].

All the experiments here presented were performed with *B. pertussis* Tohama strain since its genome was sequenced. Although some reports showed strain-dependent variation in LOS structure [37], the changes described so far in principle not affect the results achieved here since our work was focused on the comparative analysis of the immune response between the TLR4-proficient and TLR4-deficient host.

Altogether, TLR4 activation can trigger defense mechanisms in the respiratory tract, thereby regulating the early onset and outcome of the infection. The early arrival and activation of neutrophils may contribute to anti-pertussis immunity. Our results suggest that the manipulation of TLR4 signaling and neutrophil recruitment may be interesting avenues for novel therapeutic strategies against respiratory infections.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2013.06.010.

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