TLR2 deficiency by compromising p19 (IL-23) expression limits T helper 17 cell responses to *Mycobacterium tuberculosis*.

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Abstract

$CD4^+$ T helper 1 (Th1) cells producing IFN-γ are of extreme importance in controlling infections by *Mycobacterium tuberculosis* both in mice and men. In addition to IFN-γ-producing T cells, IL-17-producing T cells (Th17) have been observed during mycobacterial infections. Nevertheless, their contribution for the host immune response to mycobacteria, as well as the signals triggering *M. tuberculosis*-specific Th17 cell differentiation and maintenance are not fully understood. We show that signalling via toll-like receptor (TLR)2 has a major impact on the regulation of p19 (IL-23) expression in response to *M. tuberculosis*, and therefore on the establishment of Th17 cell responses to *M. tuberculosis* infection. Diminished Th17 responses in the lung of *M. tuberculosis*-infected TLR2 deficient animals were not caused by defective cell differentiation in the draining lymph node, but rather by reduced maintenance at the site of infection. Consistent with the decreased numbers of Th17 cells in the lungs of infected TLR2 deficient animals, we observed reduced expression of CXCL9, CXCL10 and CXCL11, chemokines involved in recall responses to *M. tuberculosis*. Our data provides insights into the TLR2 role in infection with *M. tuberculosis*, with implications in pathophysiology of the disease and vaccine design.

Introduction

Phagocytic cells, such as macrophages, dendritic cells (DC) and neutrophils are amongst the first cells to sense the presence of *Mycobacterium tuberculosis* in the host. The recognition of *M. tuberculosis* by macrophages and DC involves several pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) (1,2), DC-SIGN (3) and Dectin-1 (4). TLR2, 4 and 9 have all been shown to mediate the *in vitro* recognition of *M. tuberculosis* and the cytokine response of macrophages or DC to this pathogen is lower in the absence of these TLRs, particularly of TLR2 (5-12). As for the role of TLRs in the outcome of the infection by mycobacteria, mouse models of MyD88 deficiency showed that these animals are highly susceptible to experimental infections with *M. tuberculosis* (13-15) or with *M. avium* (16), succumbing very early upon infection. In contrast, in experimental infections with low doses of *M. tuberculosis* inocula TLR2 deficient (-/-) mice behave similarly to wild-type ones in terms of bacterial burden (7,10,12,17,18), although a defective granuloma formation was observed (7,10,17,18). When high doses of pathogen were used (7,18) or when the TLR2 deficiency was combined with TLR9 deficiency (10), a more pronounced role for TLR2 in the development of a protective immune response to *M. tuberculosis* was uncovered. In humans, several reports have linked the existence of polymorphisms in *TLR2* or TLR2-signaling molecules with susceptibility to *M. tuberculosis* (19-24) and *M. leprae* (25). However, in some populations, TLR2 polymorphisms do not associate with increased risk of tuberculosis (26). In another study, a variant of the TLR signalling adaptor protein Mal, showing an attenuated TLR2 signal transduction, was found to be protective against tuberculosis (27). Therefore, the association of *TLR2* polymorphisms with susceptibility to tuberculosis or severity of disease remains controversial and appears to greatly depend on the genetics of the host/ bacteria interplay (28).

T cell responses, in particular by IFN-γ-producing $CD4^+$ T helper 1 (Th1) cells, are of extreme importance in controlling infections by *M. tuberculosis* both in mice and men (29,30). TLR9, but not TLR2, was shown to participate in the generation of Th1 cell responses in *M. tuberculosis*-infected mice, as in its absence less IFN-γ-producing $CD4^+$ T cells were observed (10). In contrast, a more recent study showed unaffected development of Th1 cell responses in the absence of the master signalling adaptor myeloid differentiation factor 88 (MyD88) or in the combined absence of TLR2/TLR4/TLR9 (12). In addition to IFN-γ-producing T cells, IL-17-producing T cells, both γδ T cells (31-33) and Th17 cells (34-36), have been observed during mycobacterial infections. Although IL-17 appears to have a limited role in the host defence against this pathogen during primary aerogenic infection (37-39), IL-17A deficiency impacts both granuloma formation and bacterial burden (33). During vaccination against *M. tuberculosis*, Th17 cells were described to be protective by accelerating the recruitment of Th1 cells to the site of infection (40). Furthermore, deregulated production of IL-17, due to repeated exposure to mycobacterial antigen, has been recently shown to associate with extensive lung pathology (41). In humans, an increased frequency of Th17 cells was associated with latency (42), whereas, in another study, reduced Th17 cells were associated with more severe disease (43). Thus, the relative contribution of Th17 cells remains to be elucidated in human *M. tuberculosis* infection and in animal models.

The recognition mechanisms and signals that lead to IL-17 production by γδ T cells or to Th17 cell differentiation during infection by *M. tuberculosis* remain elusive. Martin et al. (44) reported that TLR2-mediated recognition of inactivated *M. tuberculosis* enhances IL-17 production by γδ T cells. A role for TLR9 in regulating *Mycobacterium bovis* BCG-elicited immune responses in mice, through the expression of delta-like 4 Notch ligand, was reported, with lower Th17 responses observed in the absence of TLR9 (45). Further understanding

these signals is important considering the role of IL-17 in vaccination, protection and pathology during mycobacterial infections. To address this issue, we investigated the role of TLR2 in the development of Th17 cell responses to *M. tuberculosis*. We found that TLR2 deficiency strongly impacts p19 (IL-23) expression during *M. tuberculosis* infection. As a result, the absence of TLR2 compromised the maintenance of *M. tuberculosis*-specific Th17 cells at the site of infection. Interestingly, the differentiation of Th17 cell in the draining lymph nodes (LN) of infected mice was not affected by the absence of TLR2. Clarifying the molecular events determining the regulation of IL-17 production by TLR2 during mycobacterial infections may provide new hints for the modulation of vaccination, protective responses and pathology.

Methods

Bacteria

H37Rv strain of *M. tuberculosis* was grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and frozen in 1ml aliquots at –80°C. Inactivated H37Ra strain of *M. tuberculosis* was purchased from Difco Laboratories.

Animals and experimental infection

Eight to 12-week-old female C57BL/6 mice, obtained from Charles River (Barcelona, Spain), C57BL/6 OT-II/rag1-/- (OT-II) TCR transgenic, obtained from the National Institute for Medical Research, or TLR2-/- mice, maintained at ICVS, were used. Mice were anesthetized with Ketamine/Medetomidine and infected intra-nasally with *M. tuberculosis* H37Rv resulting in a dose of approximately 100-200 colony forming units (CFUs) delivered into the lungs. All mouse protocols were performed according to the European Union Directive 86/609/EEC, and previously approved by the national authority *Direcção Geral de Veterinária*.

Bacterial load determination

Groups of 5-7 infected wild-type or TLR2-/- mice were killed by CO_2 asphyxiation at several time points post *M. tuberculosis* infection, the lungs were aseptically excised and homogenized in phosphate-buffered saline (PBS). Serial dilutions of the lung homogenate were plated on nutrient 7H11 agar. CFUs were counted after 3 weeks of incubation at 37°C.

Cell preparation and culture

Lungs and mediastinal LN of infected animals were aseptically removed and cell suspensions were prepared as described previously (40). Lung and mediastinal LN cells were then used for

ELISPOT, RNA analysis and flow cytometric analysis of CD4 or of GR1, as described before (34,40).

Quantitative Real Time-PCR analysis

Total RNA from infected and non-infected lungs and mediastinal LN was extracted with TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA was synthesized and analyzed by real-time PCR as described previously (46). Target gene mRNA expression was quantified using SYBR green (Qiagen) and specific oligonucleotides (Supplementary Table 1) and normalized to the ubiquitin mRNA levels or using specific primer-probes (ABI) (Supplementary Table 1) and the LightCycler-FastStart DNA Master Hybridization (Roche) and normalized to the HPRT mRNA levels.

Histological and Morphometric analysis.

Caudal lobes of lungs from wild-type or TLR2-/- infected mice were inflated with 3.8% phosphate-buffered formalin, fixed for 1 week and embedded in paraffin. Sections of 4 μm thickness were stained with hematoxylin and eosin (HE) for the inflammatory index score or used to detect iNOS by immunofluorescence with a goat anti-mouse antibody (M-19G from Santa Cruz biotechnology) and detected with Alexa Fluor 568-conjugated polyclonal rabbit anti-goat (Invitrogen). DAPI (4´,6-diamino-2-phenylindole hydrochloride) was used to counterstain tissues and to detect nuclei. All H&E sections were blindly analyzed by an independent pathologist and individually scored for inflammation according to the following scale: 0, absent; 1, mild; 2, abundant; 3, severe. Similarly, a score was attributed to the number of iNOS foci: 0, No foci; 1, 0-10 foci; 2, 10-20 foci; 3, 20-30 foci; 4, >30 foci.

154 **Statistics**

155 Data are expressed as Mean ± SEM, and the significance of differences between two means
156 was assessed with two-tailed Student t-test. Differences were considered statistically
157 significant if $p \leq 0.05$.

158

Results

TLR2 regulates Th17 cell responses to infection with *M. tuberculosis*

To clarify the molecular signals determining the establishment of *M. tuberculosis*-specific Th17 cell responses, wild-type or TLR2-/- animals were infected intra-nasally with a low dose of *M. tuberculosis*. The profile of Th cell responses was analysed by ELISPOT in the lungs of infected animals 21, 54 or 215 days post-infection. In line with previous reports (7,10,12,17,18), the bacterial burden in the lung of wild-type or TLR2-/- animals was similar (Table 1). The number of Th17 cell responses, already seemingly reduced at 21 days post-infection, was significantly diminished at days 54 and 215 post- *M. tuberculosis* infection in the absence of TLR2 (Fig. 1A). In contrast, the number of Th1 cells appeared to be unaffected by the absence of TLR2 until very late time points post-infection (Fig. 1B). We also followed the expression of IL-17A and IFN-γ in the lungs of infected animals by real-time PCR. The expression of IL-17 (Fig.1C), but not IFN-γ (data not shown), was highly affected by the absence of TLR2, being this effect more pronounced on day 54 post-infection. Consistent with the lower expression of IL-17, the transcription of chemokines known to be induced by IL-17 (40), namely CXCL9, CXCL10 and CXCL11 (Fig. 1D, 1E and 1F), was also diminished in the lung on day 54 post-infection. Our data thus suggest that TLR2 is an important upstream molecule in mediating Th17 cell responses to *M. tuberculosis*.

M. tuberculosis induced p19 expression is mediated by TLR2 signalling

Considering the importance of IL-23 for the establishment of Th17 cells, we investigated whether IL-23 expression was modulated by TLR2 recognition of *M. tuberculosis*. We measured the expression of p40 and p19, the monomers that form bioactive IL-23, in the lungs of wild-type or TLR2-/- mice during the course of infection. We observed that the induction of p19 expression observed in wild-type mice did not occur in the lungs of *M.*

tuberculosis infected TLR2-/- mice (Fig. 2A). In addition, the overall transcription of p40 in the lungs of infected animals was lower in the absence of TLR2 (Fig. 2B), whereas the expression of p35, which together with p40 forms IL-12, was not significantly affected in the absence of TLR2 (Fig. 2C). The expression of IL-6 and IL-1β in the lungs of TLR2-/- infected animals was also significantly lower than in wild-type animals at early time points post-infection (Fig. 2D and 2E). Of note, since only the cleaved IL-1β molecule is biologically active, the fact that its mRNA is downregulated in the absence of TLR2 should be interpreted with caution and only provides an indication that the amount of IL-1β may be decreased. Our data suggest that TLR2 is required for the induction of p19 and IL-1β expression, two key molecules for the establishment of appropriate *M. tuberculosis*-specific Th17 responses.

TLR2 signalling during *M. tuberculosis* infection does not impact Th17 cell differentiation in the LN

Taking into consideration that IL-23 is not involved in the initial steps of Th17 cell differentiation (47), we next investigated whether the differentiation of Th17 cells in the draining mediastinal LN during *M. tuberculosis* infection occurred normally in TLR2-/- mice. To address this issue, we started by comparing the total cell number (Fig. 3A) and the number of $CD4^+$ T cells (Fig. 3B) in the mediastinal LN of wild-type and TLR2-/- mice infected with *M. tuberculosis* for 14 or 28 days and found no differences. We chose these early time points since Th cell differentiation upon *M. tuberculosis* infection has been described to start in the mediastinal LN at around day 14 post-infection (48,49). We next assessed by ELISPOT, the number of Th17 cells in the mediastinal LN of wild-type or TLR2-/- infected animals. As shown in Fig. 3C, the number of IL-17-producing T cells in the mediastinal LN of infected animals was similar in the presence or absence of TLR2. Consistent with unaffected initiation

of Th17 responses in TLR2-/- mice, the expression of IL-6, although barely detectable in infected mediastinal LN, was not decreased in the absence of TLR2 (data not shown).

TLR2 deficiency transiently delays lung inflammation in response to *M. tuberculosis* infection

Considering that both TLR2 and IL-17 have been implicated in the inflammatory response to *M. tuberculosis*, we questioned whether TLR2 deficiency impacts the kinetics of the lung inflammatory response following *M. tuberculosis* infection. We found that at day 54 post-infection the lung inflammatory infiltration was reduced in TLR2-/- animals (Fig. 4A). However, at very late stages of infection the histological pattern observed was similar, independently on the presence or absence of TLR2 (Fig. 4A). Despite the lower amounts of IL-17 and the significantly lower inflammatory index, in TLR2-/- mice we did not observe a difference in the numbers of $GR1^+$ cells (likely neutrophils) in the lungs of TLR2-/- animals (Fig. 4B), as measured by flow cytometry. We observed that the number of iNOS-expressing foci (Fig. 4C), as assessed by immunofluorescence, was diminished in *M. tuberculosis*-infected TLR2-/- animals, 54 days post-infection. In line with the histological analysis, at late stages post-infection (215 days), both strains of animals showed similar iNOS expression (Fig. 4C). Our data thus show that TLR2 modulates the lung inflammatory response during *M. tuberculosis* infection.

Discussion

IL-17 has been recently reported as a key molecule for the development of mycobacteria granuloma (33) and lung pathology (41), in addition to its role on recall responses (40). In humans, altered Th17 cells have been associated with either latency or more severe disease (42,43). Thus, understanding the relative contribution of Th17 cells in human *M. tuberculosis* infection and in animal models is of major interest. Therefore, a better knowledge of the molecular signals needed for IL-17 expression, namely those involved in Th17 cell establishment, during *M. tuberculosis* infections is important.

TLR2 has been positively implicated as a regulator of Th17 cells in *Streptococcus pneumoniae* (50) or *Candida albicans* (51) infections, as well as in inflamed skin (52) and EAE (53), indicating that TLR2 is a key molecule on the induction of IL-17 mediated immune response. However, in infections by *Staphylococcus aureus* (54) or by *Paracoccidioides brasiliensis* (55), TLR2 acts as a negative regulator of Th17 cells. We now report that TLR2 signaling, although not required for the differentiation of *M. tuberculosis*-specific Th17 cells in the draining LN of infected animals, is of extreme importance for the induction of p19 (and thus of IL-23) and maintenance of Th17 cells at the site of infection. Our data are consistent with the well known role for IL-23 in the maintenance of Th17 cells (34,47,56), and in line with a previous report showing that mice lacking p19 produce significantly lower levels of IL-17 in the lung during *M. tuberculosis* infection (39).

In addition to inducing IL-23, we suggest that *M. tuberculosis* recognition by TLR2 may also be involved in up-regulating the expression of other factors that mediate Th17 cell responses. Consistently, in the absence of TLR2, we observed a decreased lung expression of IL-1β, another survival factor for Th17 cells (56,57). Furthermore, the expression of IL-6 in the lung

was also decreased in TLR2-/- infected mice, which can contribute via defective IL-6-trans-signalling for defective Th17 cell expansion and maintenance (58). In agreement with lower local Th17 cell responses, we also found a decreased expression of CXCL9, CXCL10 and CXCL11 in the lungs of *M. tuberculosis*-infected TLR2-/- animals. These IL-17-induced chemokines were reported to participate in the recruitment, during recall responses, of $CD4^+$ T cells producing IFN-γ, which ultimately restrict bacterial growth (40). Taking into consideration that *M.tuberculosis*-specific Th17 cells, following vaccination, are mainly resident in the lung and contribute to a faster recruitment of protective Th1 cells (40), signals that sustain Th17 responses, including as we now show those mediated by TLR2, may be of major interest to modulate the local immune response and potentiate vaccine efficacy.

Our study, placing TLR2 signaling as an important molecular mediator of effective Th17 cell responses during *M. tuberculosis* infections, unveils a potential role for TLR2 signals during recall responses, such as during vaccination (an hypothesis that we are currently addressing) or re-infection. This is of particular importance as, in humans, polymorphisms in the *TLR2* gene have been associated to increased severity of tuberculosis (19-24). Since most of the human studies have been performed in endemic areas, it is tempting to speculate that the problems faced by these individuals to control tuberculosis might be associated with impairment in mounting appropriate recall responses.

Previous studies suggested that mice lacking TLR2 suffered an exacerbated pathology in the lungs upon infection with *M. tuberculosis*, which was related to defective granuloma formation (10,17,18). In our study, we observed a transient delay in the lung inflammatory response of TLR2-/- mice, as assessed by histological analysis and quantification of iNOS-expressing foci. At late times post-infection, however, wild-type and TLR2-/- animals showed

similar patterns of lung and liver (data not shown) inflammation, suggesting that the observed defect is transient and can be compensated over-time. It is conceivable that, in situations where TLR2-/- mice are unable to compensate the delayed inflammatory response in the lung, as it is the case of infection with high doses of inoculum (7,18) or of combined TLR2/9 deficiencies (10), the control of bacterial growth may be put at risk.

Lung pathology during *M. tuberculosis* infection frequently associates with increased neutrophil influx and formation of pyogranuloma. TLR2-/- mice have been shown previously to have increased lung neutrophil influx (10,17,18). We did not observe a difference in the number of $GR1^+$ cells (likely neutrophils) in the lungs of *M. tuberculosis*-infected TLR2-/- animals. This discrepancy could be due to the fact that different routes and doses of infection were used. Of note, in a model of *M. avium* intra-venous infection, TLR2-/- animals did not exhibit an enhanced influx of neutrophils into the liver (16).

Understanding the molecular signals that dictate Th17 differentiation during infection gains a novel importance in light of the recent evidence for a role of IL-17 in determining protection versus pathology during infections by mycobacteria (33,41-43) Furthermore, considering the described role of Th17 cells in accelerating Th1 responses during secondary infections (40), and thus in increasing the efficacy of vaccination, TLR2 deficient signals, as our results now suggest, may compromise the efficiency of vaccination against tuberculosis and provide new targets for vaccine improvement.

Abbreviations

CFU, colony forming units; CXCL, CXC ligand; HE, hematoxylin and eosin; IFN, Interferon; IL, Interleukin; LN, lymph nodes; MyD88, myeloid differentiation factor 88; OVA ovalbumin; PRR, pattern recognition receptor; Th, T helper; TLR, Toll like receptor; -/-, deficient.

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Table 1. Bacterial counts (Mean±SEM) in the lungs of wild-type or TLR2-/- mice infected with *M. tuberculosis*[a].

	Bacterial counts (log_{10}CFU)	
	Wild-type	**TLR2-/-**
Day 1	2.28±0.05	2.10±0.11
Day 50	5.17±0.42	4.71±0.15
Day 215	5.33±0.30	4.69±0.28

[a] At the indicated time points, lungs from 5-6 infected mice were harvested and homogenized, diluted and then plated to determine the number of mycobacterial colony forming units per organ. Means were determined to be not statistically different as described in the methods.

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Figure legends

Figure 1: Lung Th17 cell responses during infections by *M. tuberculosis* are compromised in the absence of TLR2.

Wild-type or TLR2-/- mice were infected intra-nasally and, at the indicated time points, lung cell suspensions were prepared, isolated cells stimulated for 24h with ESAT-6 peptide presented by total spleen irradiated antigen-presenting cells and IL-17 (A) or IFN-γ (B) production assessed by ELISPOT. In parallel, RNA was extracted from the lung tissue and the expression of IL-17 (C), CXCL9 (D), CXCL10 (E) or CXCL11 (F) analyzed by quantitative real-time PCR and normalized to the expression of ubiquitin (C) or HPRT (D-F). Data represented for day 0 correspond to uninfected animals. Data points show the Mean ± SEM value for 5-6 mice per group and the significance was determined by the Student´s t test (*$p<0.05$; **$p<0.01$). The data are representative of two independent experiments.

Figure 2: TLR2 regulates the expression of p19 in the lung, during *M. tuberculosis* infections.

Wild-type or TLR2-/- mice were infected as before and at the indicated time points, lung tissue was obtained, RNA extracted and p19 (A), p40 (B), p35 (C), IL-6 (D) and IL-1β (E) expression measured by real-time PCR and normalized to ubiquitin. Data points show the Mean ± SEM value for 5-6 mice per group and the significance was determined by the Student´s t test (*$p<0.05$). Data represented for day 0 correspond to uninfected animals. The data are representative of two independent experiments.

Figure 3: Absence of TLR2 does not affect the frequency of Th17 cells in the LN of *M. tuberculosis*-infected animals.

Wild-type or TLR2-/- mice were infected as in Fig.1 and, on days 14 and 28 post-infection, mediastinal LN were harvested, cell suspensions obtained and counted for total cell number determination (A) or stained with anti-CD4-APC specific antibodies for $CD4^+$ cell number determination by flow cytometry (B). Cells were also stimulated as indicated in Fig. 1 for the assessment of IL-17 production by ELISPOT (C). Data points show the Mean ± SEM value for 5-6 mice per group and the significance was determined by the Student´s t test. The data are representative of two independent experiments.

Figure 4: TLR2 regulates lung inflammation during *M. tuberculosis* infections.

Wild-type or TLR2-/- mice were infected as before and, at the indicated time points post-infection, sections were prepared from formalin-fixed lungs. The degree of inflammation in the lungs of multiple mice was quantified in a blinded manner using a scale (the inflammatory indexes were: 0, absent; 1, mild; 2, abundant; 3, severe inflammation). The values from independent lungs were then combined to give a mean±SD (A). At the indicated time points, lung cell suspensions were prepared and analyzed by flow cytometry for surface expression of GR1 (B). The number of iNOS-expressing foci in the lung tissue was determined by immunofluorescence, quantified and scored for each animal within the group (5-6 animals). iNOS foci scores were: No foci=0; 0-10=1; 10-20=2; 20-30=3; >30=4 (C). Significance was determined by the Student´s t test (*$p<0.05$). The data are representative of two independent experiments.

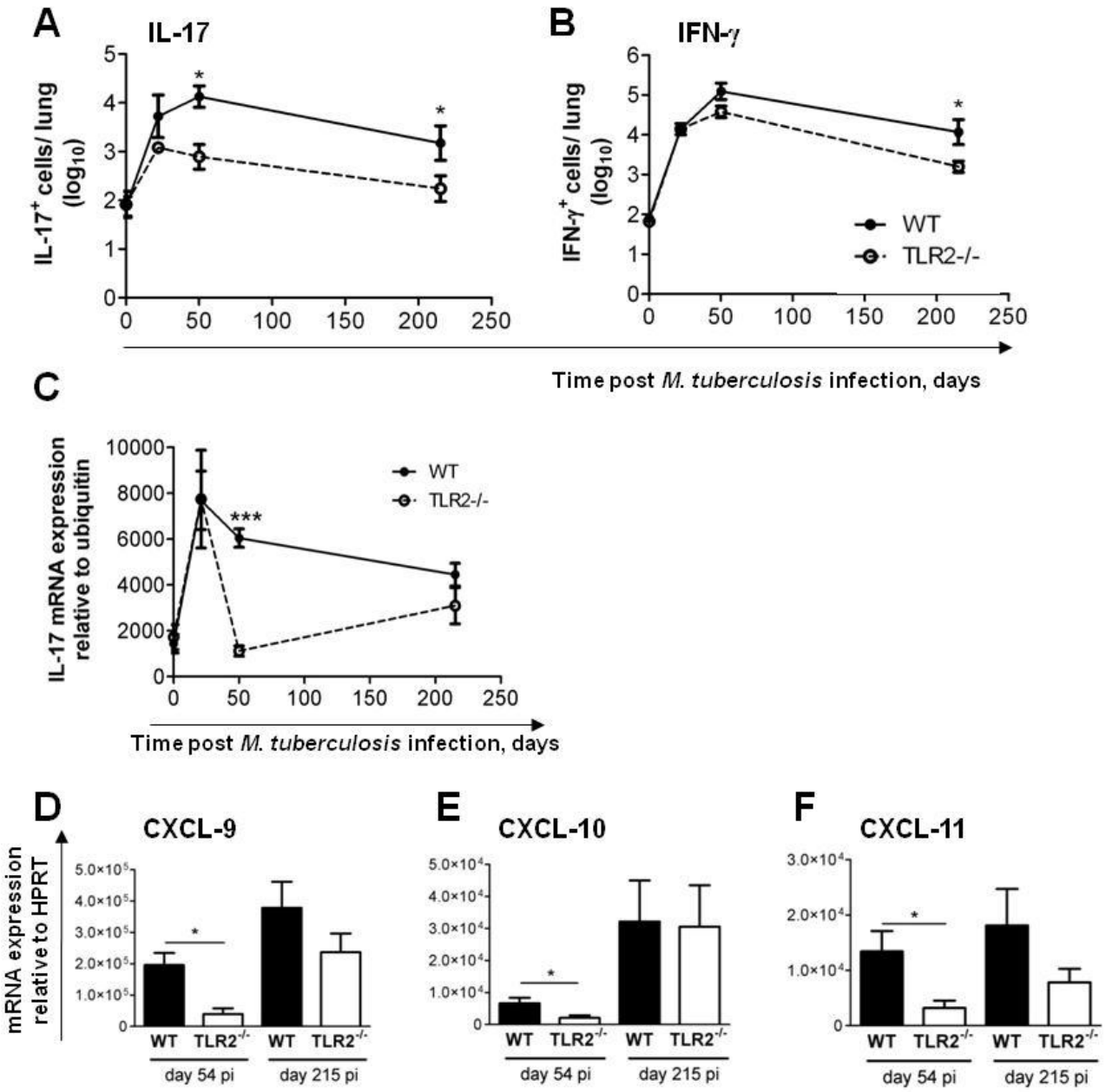

Figure 1
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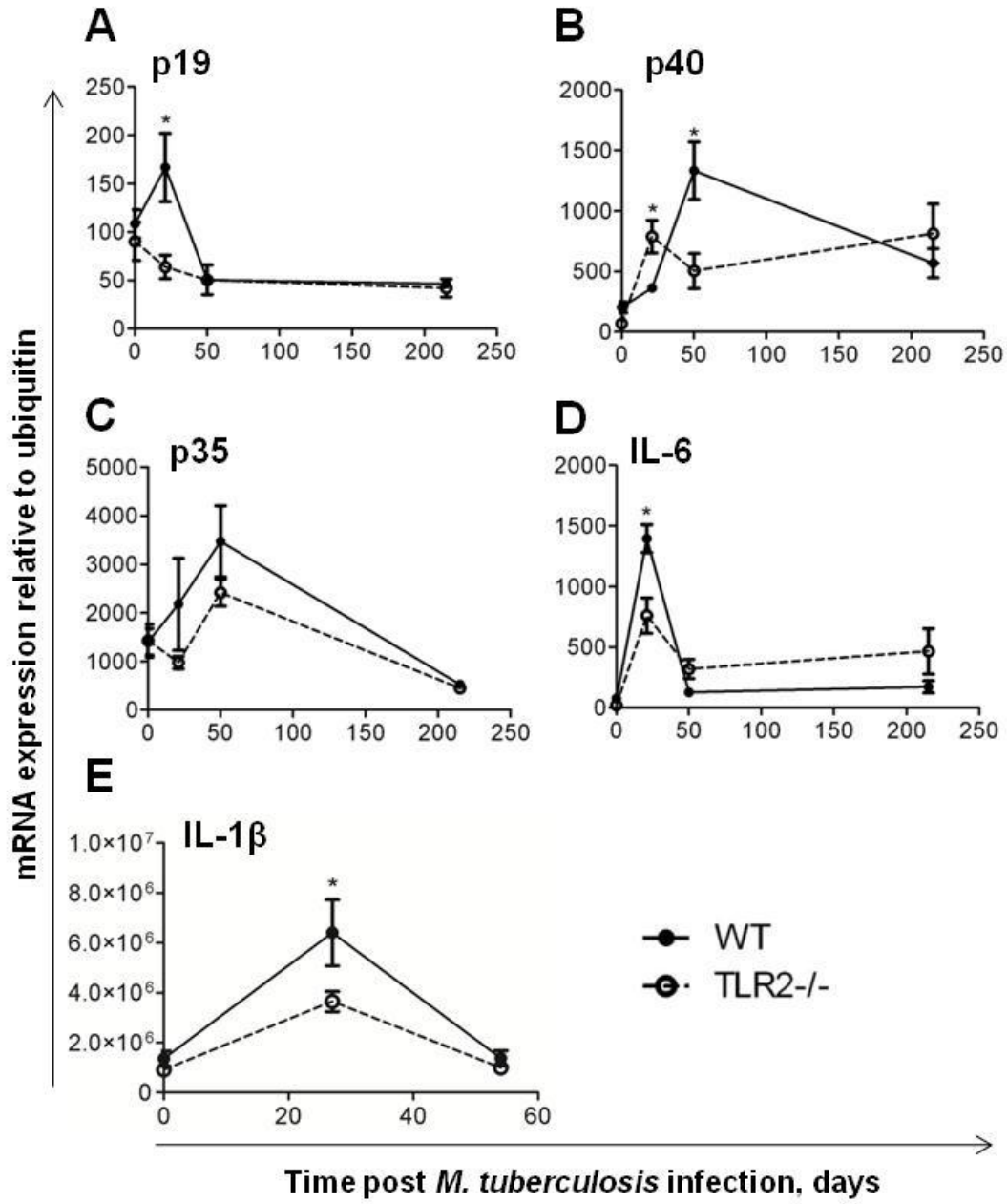

Figure 2
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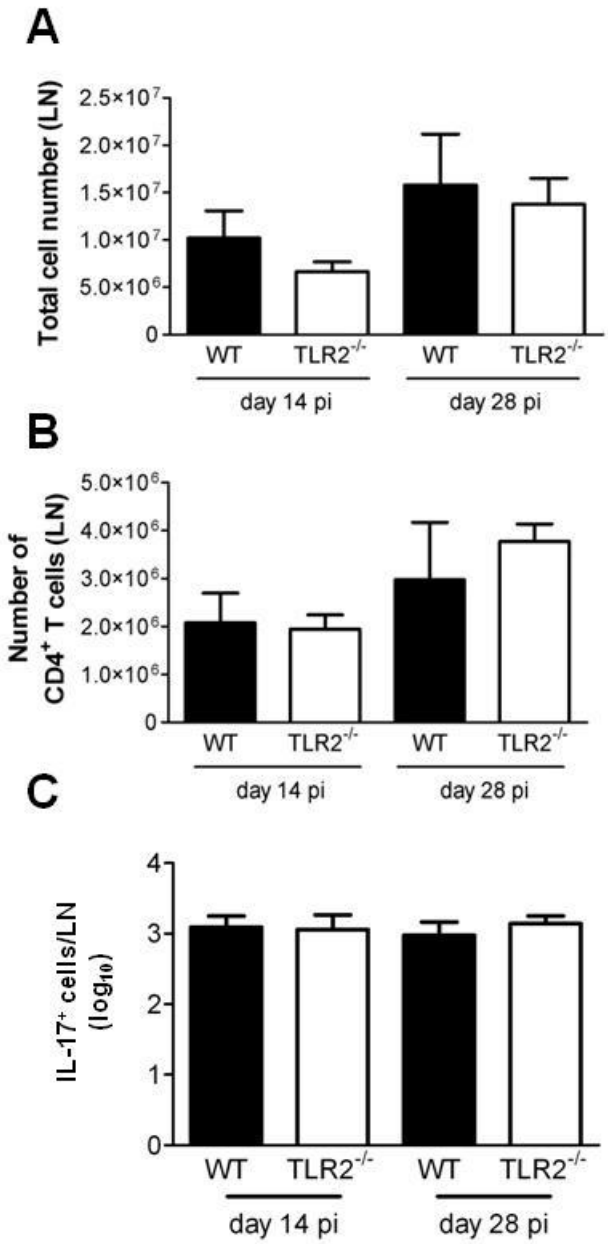

Figure 3
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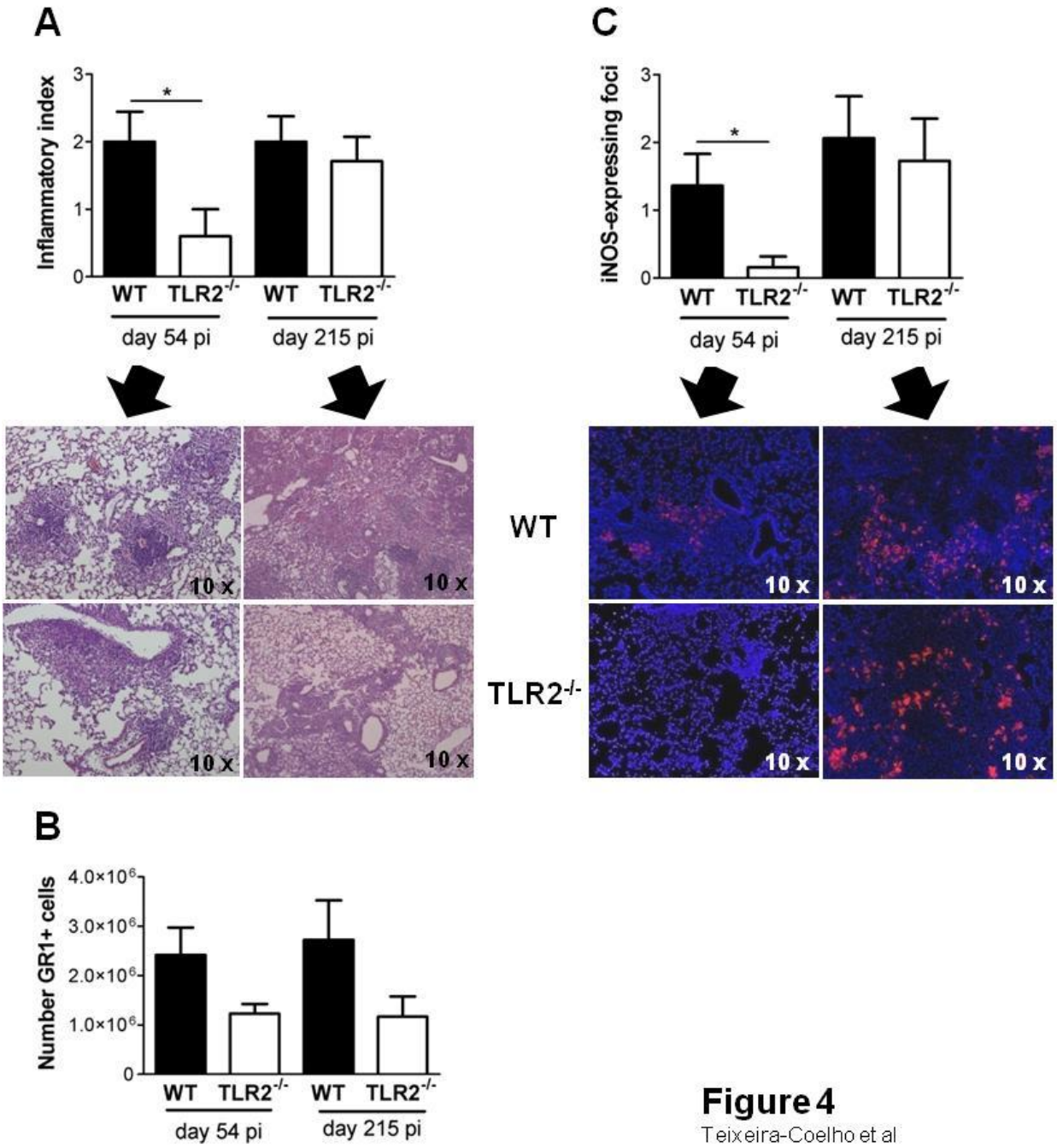

Figure 4
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587 **Supplementary Table I: Sequences of the specific oligonucleotides and references of the primer-probe sets**

588 **used for mRNA quantification by real-time PCR.**

Gene	**Forward oligonucleotide (5'→3')**	**Reverse oligonucleotide (5'→3')**
IL-12p40	CAA ATT ACT CCG GAC GGT TC	AGA GAC GCC ATT CCA CAT GTC
IL-23p19	CGT ATC CAG TGT GAA GAT GGT TGT	GCT CCC CTT TGA AGA TGT CAG A
IL-12p35	TGC TGG TGG CCA TCG AT	GCA GAG TCT CGC CAT TAT GAT T
IL-17	CTC AGA CTA CCT CAA CCG TTC CA	TTC CCT CCG CAT TGA CAC A
IFN-γ	CAA CAG CAA GGC GAA AAA GG	GGA CCA CTC GGA TGA GCT CA
IL-6	ACA CAT GTT CTC TGG GAA ATC GT	AAG TGC ATC ATC GTT GTT CAT ACA
IL-1β	ACC TTC CAG GAT GAG GAC ATG A	AAC GTC ACA CAC CAG CAG GTT A
Ubiquitin	TGG CTA TTA ATT ATT CGG TCT GCA T	GCA AGT GGC TAG AGT GCA GAG TAA
Gene	Primer-probe set reference (Applied Biosystems)	
CXCL9	Mm00434946_m1	
CXCL10	Mm 99999072_m1	
CXCL11	Mm00444662_m1	
HPRT	Mm00446968_m1	

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