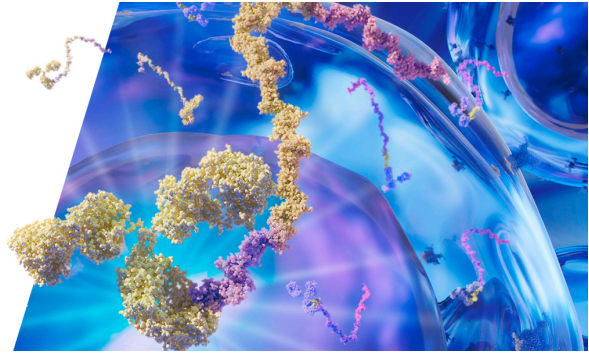


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### IL-6 and IL-10 Induction from Dendritic Cells in Response to *Mycobacterium tuberculosis* Is Predominantly Dependent on TLR2-Mediated Recognition<sup>1</sup>

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# IL-6 and IL-10 Induction from Dendritic Cells in Response to *Mycobacterium tuberculosis* Is Predominantly Dependent on TLR2-Mediated Recognition<sup>1</sup>

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The initial TLR-mediated interaction between *Mycobacterium tuberculosis* and dendritic cells is critical, since the cytokine production that ensues can greatly influence the class of adaptive immunity that is generated to the pathogen. In this study, we therefore determined the dependency on TLR2 and TLR4 for *M. tuberculosis*-induced cytokine production by murine dendritic cells. A key new finding of this study is that production of IL-6 and IL-10 from dendritic cells in response to *M. tuberculosis* is principally dependent on TLR2. The study also indicates that *M. tuberculosis* can induce IL-12 production in the absence of either TLR2 or TLR4, suggesting redundancy or possibly involvement of other receptors in IL-12 production. In addition, the data also reveal that lack of TLR2 or TLR4 does not impact on dendritic cell maturation or on their ability to influence the polarity of differentiating naive T cells. Collectively, data presented here provide a mechanistic insight for the contribution of TLR2 and TLR4 to tuberculosis disease progression and offer strategies for regulating IL-6 and IL-10 production in dendritic cell-based vaccine strategies. *The Journal of Immunology*, 2004, 173: 3392–3397.

An extensive body of literature now exists which clearly places the TLRs as key players in innate immune recognition of conserved molecular patterns expressed on microbes (1–5). To date, 10 homologues of TLRs have been reported, and ligands for most of the TLR members have been recognized (6, 7). For example, lipoproteins, peptidoglycans, and lipoarabinomannan are TLR2 agonists and LPS binds to TLR4 (8, 9), and, on the contrary, TLR9 is activated by bacterial DNA (10). Ligation of TLRs initiates a signal transduction pathway in the host cell that culminates in the activation of NF- $\kappa$ B and induction of several immune-related genes, including cytokines and chemokines (11, 12). Thus, TLR activation is an important link between innate cellular responses and the subsequent activation of adaptive immune response to microbial pathogens.

Human tuberculosis is one of the most rampant infectious diseases and a leading cause of death worldwide (13). Cell-mediated immune responses are protective in tuberculosis, and production of cytokines and chemokines by macrophages and dendritic cells is crucial to initiating cellular immune responses to *M. tuberculosis*, the causative agent of the disease (14). Given the fact that *M. tuberculosis* infects professional phagocytes that express a diverse array of TLRs, it is not surprising that a number of groups are intensely examining the interaction between TLRs and their ligands present in *M. tuberculosis*. Data from several studies now

demonstrate that both TLR2 and TLR4 recognize several different secreted *M. tuberculosis* products to initiate cellular activation. The 19-kDa lipoprotein, a secreted Ag of *M. tuberculosis*, interacts specifically with TLR2 to induce TNF- $\alpha$  and NO production from both murine and human macrophages (15). In addition, the 19-kDa lipoprotein is also a major inducer of IL-12 production in human monocytes (15). Two other secreted ligands, phosphatidylinositol-mannan (PIM) and a soluble tuberculosis factor have also been shown to interact with TLR2 (16). However, with regard to PIMs, Abel et al. (17) demonstrated that PIM structures can also elicit cellular activation via TLR4. They showed that PIM was able to induce NF- $\kappa$ B activation in a dose-dependent manner in stable TLR4 and MD-2 Ba-F3 transformants. Heat-sensitive membrane-associated factors of *M. tuberculosis* have also been implicated to function as TLR4 agonists (18). Interestingly, mannose-capped lipoarabinomannan derived from virulent *M. tuberculosis* fails to activate either TLR2 or TLR4-transfected cells (19). In contrast, nonmannose-capped lipoarabinomannan purified from fast-growing mycobacteria is capable of TLR-2 mediated cellular activation (19).

In contrast to studies with secreted products of *M. tuberculosis*, only a few studies have addressed the outcome of TLR interaction with whole *M. tuberculosis*. Means et al. (18) reported that the avirulent strain of mycobacteria H37Ra contain distinct ligands that activate RAW 264.7 macrophages via TLR2 and TLR4. Specific blocking of TLR4 resulted in a reduction in TNF- $\alpha$  production, indicating a substantial TLR4 involvement in *M. tuberculosis*-induced TNF- $\alpha$  secretion (20). In contrast, another study reported that expression of an inhibitory TLR2 in RAW TT10 macrophage cells completely abrogated TNF- $\alpha$  production by *M. tuberculosis* H37Rv and H37Ra strains (21), indicating that TLR2 is the principle mediator of TNF- $\alpha$  production by whole *M. tuberculosis*.

Based on these in vitro data indicating that *M. tuberculosis* and its secreted products interact with TLR2 and TLR4 led investigators to determine the contribution of the two TLRs in host resistance to disease. Two groups looked at the requirement of TLR4 in controlling *M. tuberculosis* infection following an aerosol challenge. Both groups used the TLR4 mutant C3H/HeJ and compared

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it to the wild-type C3H strain. Ryffel's group (17) reported that TLR4 expression was required to control chronic *M. tuberculosis* infection, and in contrast Behar's group (22) showed that TLR4 mutant mice are not any more susceptible than other C3H substrains. These observed differences could be attributable to differing infection doses that were used in the two studies. The former study (17) used a very high inoculum for aerosol challenge, while the latter study (22) used a low-dose aerosol challenge. Yet another study (23) using TLR2 and TLR4 gene-deficient mice and a model of low-dose aerosol infection demonstrated that lack of either TLR2 or TLR4 did not affect the outcome of *M. tuberculosis* infection. However, in this study (23), high-dose aerosol challenge did reveal a role for TLR2 but not TLR4 in host resistance. Recently Akira's group (24) also showed that TLR2-deficient mice exhibited similar pulmonary pathology to that observed in wild-type mice.

It is clear that dendritic cells and the cytokines they produce in response to a pathogen will regulate the ensuing adaptive immune response to the pathogen. We argued that the cytokine profile of dendritic cells following *in vitro* infection would reflect the cytokine milieu that is generated early on *in vivo*, in response to *M. tuberculosis* infection. We therefore decided that it may be a valuable undertaking to carry out a systematic *in vitro* analysis of the dependency on TLR2 and TLR4 for *M. tuberculosis*-induced cytokine production by dendritic cells. We also deliberated that the *in vitro* studies may help better understand the differing outcomes seen in the *in vivo* studies. *In vivo* studies give end results that also involve soluble ligands of TLRs generated following infection and *M. tuberculosis* replication within host cells. Involvement of TLR and whole *M. tuberculosis* early on in cytokine production from dendritic cells and its affect on the class of adaptive immune response generated is important in vaccine design where there is not a significant replication of bacteria and the initial interaction between *M. tuberculosis* and dendritic cells is critical.

## Materials and Methods

### Mice

BALB/c, C57BL/6, C.C3-*Tlr4*<sup>Lps-d</sup> (TLR4 mutant), and OVA peptide-specific DO11.10 TCR-transgenic mice on the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME). The C3H/HeJ strain of mice is the original strain that was shown to have defective LPS responsiveness due to mutations in *Tlr4* (8). These mice were backcrossed onto BALB/c background at The Jackson Laboratory and the mutant mice on the BALB/c background are referred to as C.C3-*Tlr4*<sup>Lps-d</sup>. TLR2-deficient mice (25) on the C57BL/6 genetic background were provided to us by Dr. H. Shen (University of Pennsylvania, Philadelphia, PA). TNF- $\alpha$ -deficient mice on the C57BL/6 genetic background (The Jackson Laboratory) were provided to us by Dr. J. Chan (Albert Einstein College of Medicine, New York, NY). All strains of mice were maintained under pathogen-free conditions at the Rodent Barrier Facility of Temple University School of Medicine (Philadelphia, PA).

### Mycobacteria

Frozen stocks of the virulent *M. tuberculosis* Erdman strain (Trudeau Institute, Saranac Lake, NY) was kindly provided by Dr. J. Chan (Albert Einstein College of Medicine). The strain obtained after mouse passage was grown in culture, titrated, and stored at  $-70^{\circ}\text{C}$ . Before infection, aliquots were thawed, briefly sonicated, and then added to cultures at the appropriate multiplicity of infection (MOI).<sup>4</sup> Irradiated H37Rv strain was provided by Dr. J. Belisle (Colorado State University, Fort Collins, CO).

### Isolation of transgenic T cells

Naive transgenic T cells were isolated from the spleens of DO11.10-transgenic mice. After lysis of RBC with 0.147 M Tris-NH<sub>4</sub>Cl (pH 7.0), T cells

were purified by removal of macrophages and B cells by plastic adherence and followed by adherence to nylon wool columns (Polysciences, Warrington, PA) (26)

### Dendritic cell preparation

Bone marrow-derived dendritic cells (BMDCs) were prepared as previously described (27). Briefly, bone marrow was flushed out from the femur and tibia and  $2 \times 10^6$  of bone marrow cells were seeded into 10-cm petri dishes in 10 ml of RPMI 1640 containing 10% FCS (HyClone, Logan, UT) and supplemented with penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), glutamine (2 mM), 2-ME (50 mM), and 20 ng/ml murine rGM-CSF (PeproTech, Rocky Hill, NJ). On day 3, an additional 10 ml of complete medium containing rGM-CSF was added to the cultures. On days 6 and 8, the cultures were fed by changing 50% of the medium. Nonadherent cells were harvested on day 10. Resultant nonadherent cells were typically  $>70\%$  CD11c<sup>+</sup>CD11b<sup>+</sup> as determined by FACS analysis.

### Cytokine assays

The presence of cytokines in the supernatants was determined by sandwich ELISA using the following Ab pairs from BD Pharmingen (San Diego, CA): C15.6 and C17.8 (biotinylated) for IL-12p40; JES5-2A5, and JES5-16E3 (biotinylated) for IL-10; R4-6A2, and XMG1.2 (biotinylated) for IFN- $\gamma$ ; 11B11 and BVD6-24G2 (biotinylated) for IL-4; G281-2626, and MP6-XT3 (biotinylated) for TNF- $\alpha$  and 9A5 and C17.8 (biotinylated) for IL-12p70. OptEIA mouse IL-6 kit (BD Pharmingen) was used for IL-6 measurements.

### Flow cytometry

Cell surface expression of B7.1, B7.2, and CD40 was measured by flow cytometry following labeling of cells with FITC-conjugated anti-mouse B7.1 Ab, PE-conjugated anti-mouse B7.2 Ab, and FITC-conjugated anti-mouse anti-CD40 Ab (clone HM40-3; BD Pharmingen), respectively. The anti-B7.1 (IG10) and anti-B7.2 (2D10) Abs have been described previously (28) and were kindly provided by Dr. V. Kuchroo (Brigham and Women's Hospital, Boston, MA). Data are expressed as the percentage of positive cells and as the mean fluorescence intensity (MFI).

### Detection of NF- $\kappa$ B p65

Nuclear extracts were prepared as previously described (29), with slight modifications that included addition of leupeptin (5  $\mu\text{g}/\text{ml}$ ) and aprotinin (5 mg/ml) to both the hypotonic and lysis buffers. Twenty-five micrograms of nuclear extract was resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The blots were probed with anti NF- $\kappa$ B p65 Ab (C-20 Ab from Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated donkey anti-rabbit IgG Ab (Amersham Biosciences). Blots were developed using the ECL Plus system (Amersham Biosciences).

### Statistics

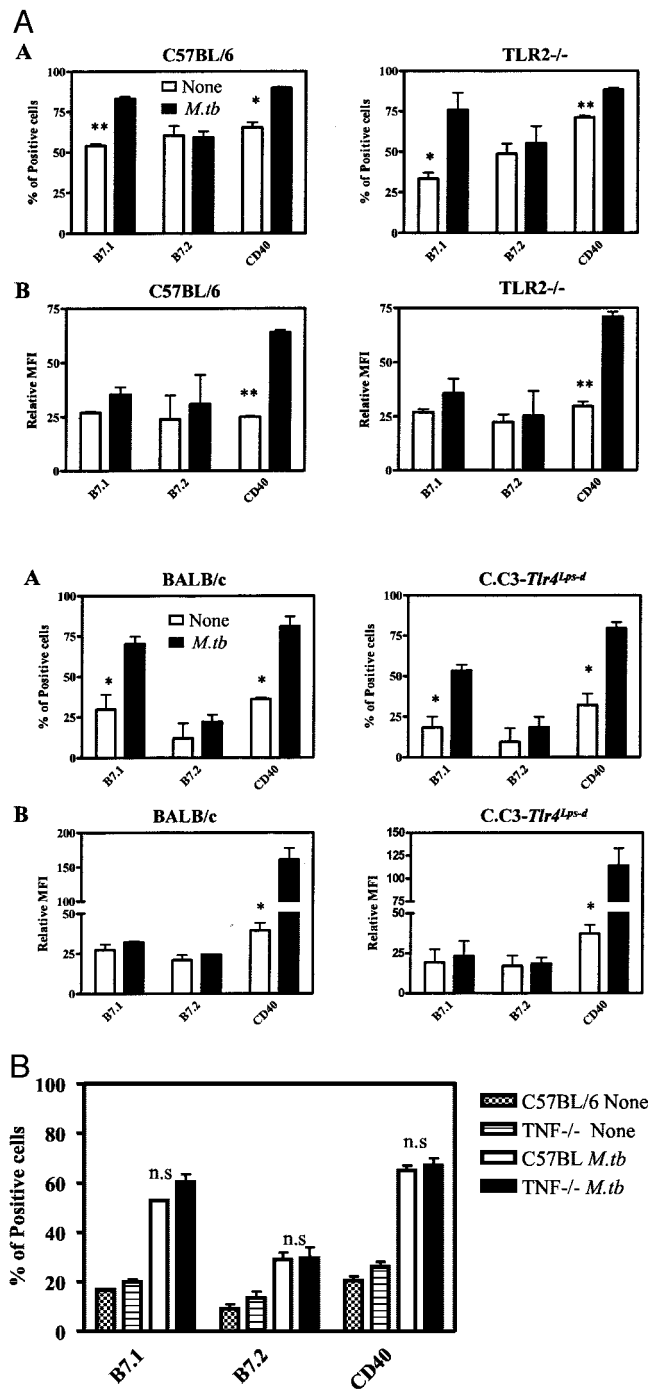
For statistical analysis, unpaired Student's *t* test was performed (PRISM version 4.0; GraphPad, San Diego, CA).

## Results

### Contribution of TLR2 and TLR4 to dendritic cell maturation in response to *M. tuberculosis*

BMDCs derived from TLR2<sup>-/-</sup>, C.C3-*Tlr4*<sup>Lps-d</sup>, C57BL/6, and BALB/c mice were examined for their ability to mature following *M. tuberculosis* infection. Maturation of BMDCs was determined by comparing the levels of cell surface expression of B7.1, B7.2, and CD40 between uninfected and infected cells. In the four groups of mice examined, a constitutive expression of all three cell surface markers of maturation was observed. Comparison of these maturation markers between C57BL/6 and TLR2-deficient mice following *M. tuberculosis* infection revealed no significant differences in the expression profile. As shown in Fig. 1A, percentage of cells expressing B7.1 and CD40 was significantly up-regulated in both the wild-type and the TLR2-deficient BMDCs. However, both groups did not show any enhancement in B7.2 expression following infection. Similarly, when MFIs were compared between the two groups following infection, a significant increase in MFI for only CD40 was observed in both groups. Correspondingly, when BMDCs from BALB/c and the TLR4 mutant were

<sup>4</sup> Abbreviations used in this paper: PIM, phosphatidylinositolmannan; MOI, multiplicity of infection; BMDC, bone marrow-derived dendritic cell; MFI, mean fluorescence intensity.



**FIGURE 1.** Maturation of *M. tuberculosis*-infected BMDCs from TLR2<sup>-/-</sup> and TLR4-defective mice. **A**, BMDCs were infected with *M. tuberculosis* for 24 h. Expression of surface markers on BMDCs was examined by labeling with fluorescently tagged anti-B7.1, anti-B7.2, and anti-CD40 Abs followed by flow cytometric analyses. Data are expressed as the percentage of positive cells (**A**) and as the MFI of B7.1, B7.2, or CD40-positive cells. \*,  $p < 0.05$  and \*\*,  $p < 0.01$ . **B**, BMDCs from wild-type C57BL/6 and TNF<sup>-/-</sup> mice were infected with *M. tuberculosis* for 24 h. Expression of surface markers on BMDCs was examined as described in **A**.

compared, we observed no significant differences in the pattern of up-regulation of the three maturation markers following infection with *M. tuberculosis*. As seen in the previous comparison, there was a significant increase in B7.1 and CD40 expression following *M. tuberculosis* infection. Interestingly, both of these strains had a low-level constitutive expression of B7.2. This difference could

probably be due to inherent genetic differences that exist between C57BL/6 and BALB/c strains. With regard to MFI, increased MFI was again observed only in CD40. Together, these data indicate that absence of either TLR2 or TLR4 signaling had no impact on the ability of BMDCs to mature in response to *M. tuberculosis* infection.

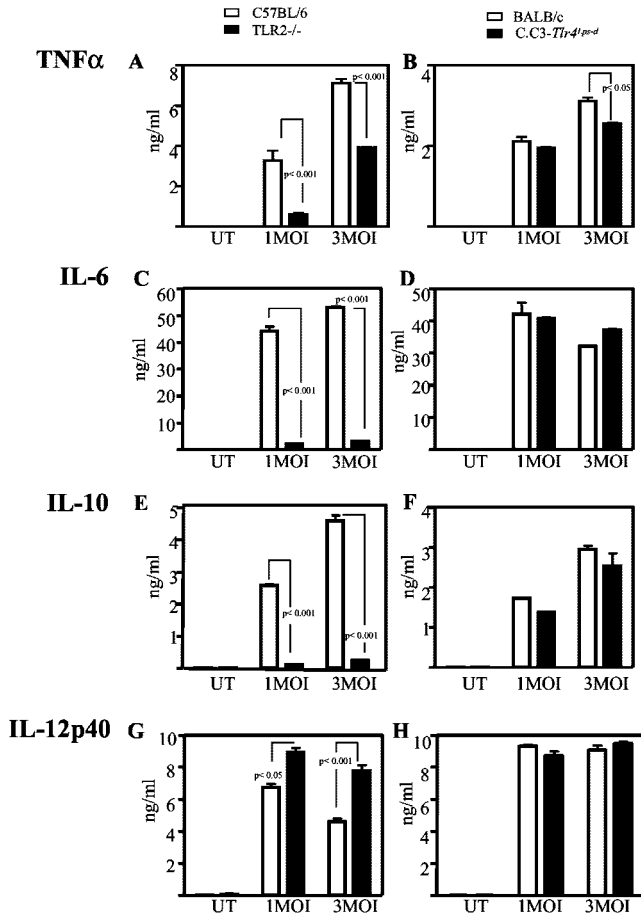
Since TNF- $\alpha$  can induce maturation of dendritic cells (30, 31), we wanted to determine in our system whether *M. tuberculosis*-induced dendritic cell maturation was dependent on TNF- $\alpha$ . As shown in Fig. 1B, following infection with *M. tuberculosis* BMDCs from both wild-type C57BL/6 and TNFKO mice had a similar increase in the percentage of cells expressing B7.1, B7.2, and CD40. These data indicate that *M. tuberculosis* induces dendritic cell maturation independent of TNF- $\alpha$ . We therefore did not further pursue the role of this cytokine in maturation of TLR2- and TLR4-deficient dendritic cells.

#### The role of TLR2 and TLR4 in the production of cytokines by dendritic cells in response to *M. tuberculosis*

Previously, we had reported that BMDCs release TNF- $\alpha$ , IL-12, and IL-10 following infection with *M. tuberculosis* (32). In this study, we determined the distinct participation of TLR2 and TLR4 to the overall dendritic cell cytokine secretion profile in response to stimulation with virulent whole *M. tuberculosis*. Treatment with *M. tuberculosis* of BMDCs from wild-type C57BL/6 and TLR2<sup>-/-</sup> mice resulted in differences in the expression pattern of three of the four cytokines examined. As shown in Fig. 2A, TNF- $\alpha$  production was markedly reduced in BMDCs from TLR2<sup>-/-</sup> mice at 1 MOI. Interestingly, at 3 MOI the levels of TNF- $\alpha$  increased substantially in both the wild-type and the TLR2-deficient BMDCs, although there was a significantly lower production of the cytokine in the absence of TLR2 signaling. These data suggest a partial dependency on TLR2 for TNF- $\alpha$  production by BMDCs to *M. tuberculosis*. A differential induction of IL-6 and IL-10 production was also observed between C57BL/6 and TLR2<sup>-/-</sup> BMDCs (Fig. 2, C and E). In contrast to TNF- $\alpha$  release, the absence of TLR2 signaling significantly abrogated the secretion of both IL-6 and IL-10. This significant reduction in IL-6 and IL-10 production was seen at both 1 and 3 MOIs, indicating a huge dependency by BMDCs on TLR2 signaling for the production of these two cytokines in response to *M. tuberculosis* infection. Surprisingly though, IL-12p40 production was seen in BMDCs derived from both wild-type and TLR2<sup>-/-</sup> mice (Fig. 2G). Moreover, worthy of note, there was in fact a modest but significant increase in IL-12p40 production in the absence of TLR2 signaling.

To investigate the contribution of TLR4 to *M. tuberculosis*-induced cytokine production, we compared the production of cytokines from BMDCs derived from wild-type BALB/c mice and the TLR4 mutant C.C3-Tlr4<sup>Lps-d</sup> mice that is defective in TLR4 signaling. Intriguingly, lack of TLR4 did not result in significant differences in the secretion of either TNF- $\alpha$  (Fig. 2B), IL-6 (Fig. 2D), IL-10 (Fig. 2F), or IL-12p40 (Fig. 2H). However, it is worth noting that following 3 MOI there was decreased TNF- $\alpha$  secretion from the TLR4 mutant BMDCs. These data connote that there is minimal contribution from TLR4 to the immediate cytokine release by BMDCs in response to *M. tuberculosis*.

The similar induction of IL-12p40 in the absence or presence of TLR2 and TLR4 signaling prompted us to determine the levels of the biologically active IL-12p70. Fig. 3A shows that akin to IL-12p40, BMDC expression of IL-12p70 was also equivalent between C57BL/6 and TLR2<sup>-/-</sup> mice. Comparable levels of IL-12p70 were also released from BMDCs obtained from BALB/c and the TLR4 mutant mice (Fig. 3B). These data suggest that the production of the Th1-polarizing cytokine, IL-12 from BMDCs in



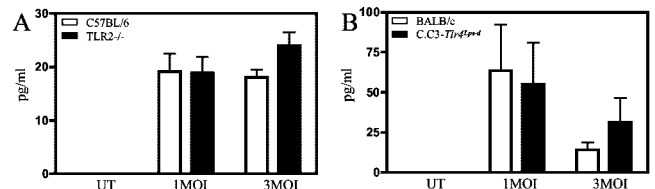
**FIGURE 2.** Differential cytokine production from TLR2<sup>-/-</sup> and TLR4-defective dendritic cells. BMDCs were stimulated with *M. tuberculosis* at varying MOIs. After 24 h, supernatants were harvested and analyzed for the presence of TNF-α (A and B), IL-6 (C and D), IL-10 (E and F), and IL-12p40 (G and H) by sandwich ELISA. A, C, E, and G, Comparisons between BMDCs derived from wild-type C57BL/6 and TLR2<sup>-/-</sup>. B, D, F, and H, Comparisons between BMDCs derived from wild-type BALB/c and C.C3-Tlr4<sup>Lps-d</sup> mice.

response to *M. tuberculosis* is both TLR2 and TLR4 independent or alternatively there is redundancy of signaling and *M. tuberculosis* can engage either TLR2 or TLR4 for IL-12 production.

In some of these experiments, we included polymyxin at 10 μg/ml concentration along with *M. tuberculosis* and found that the cytokine responses did not differ from those induced by *M. tuberculosis* stimuli alone (data not shown). This rules out the possibility that signaling from LPS occurred in this system.

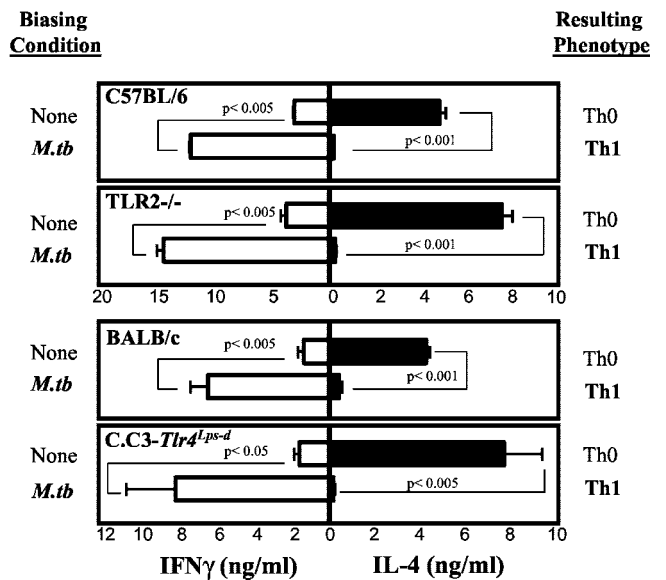
*Lack of either TLR2 or TLR4 does not affect the Th1-polarizing ability of M. tuberculosis-infected dendritic cells*

To further examine the issue that both TLR2-deficient and TLR4-defective BMDCs can produce bioactive IL-12 following *M. tuberculosis* infection, we tested the in vitro priming ability of the two groups of dendritic cells. For these experiments, BMDCs were infected at 1 MOI for 24 h. Naive T cells obtained from the spleens of DO11.10-transgenic mice were then allowed to differentiate in the presence of OVA peptide (0.3 μM), irradiated DO11 splenocytes, and the *M. tuberculosis*-infected dendritic cell cytokine milieu for 72 h. T cells were then re-exposed to Ag and APCs in the absence of any biasing modalities. Cytokine profile of the differentiated T cells was then determined by ELISA. As previously noted by us (32), in the absence of *M. tuberculosis* infection, den-

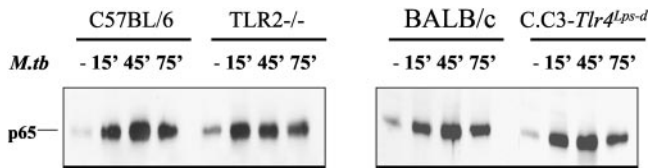


**FIGURE 3.** Both TLR2<sup>-/-</sup> and TLR4-defective BMDCs can produce bioactive IL-12 following *M. tuberculosis* infection. BMDCs obtained from TLR2<sup>-/-</sup> mice (A) and TLR4-defective mice (B) were infected with *M. tuberculosis*. After 24 h, supernatants were harvested and analyzed for IL-12p70 by sandwich ELISA.

dritic cells had no polarizing effect on the differentiating naive T cells. However, the naive T cells acquired Th1 polarity when allowed to differentiate in the presence of *M. tuberculosis*-infected dendritic cells obtained from either C57BL/6 or BALB/c mice, as evidenced by a significant decrease in IL-4 levels and a concomitant increase in IFN-γ levels (Fig. 4). It has been previously suggested that TLR2 signaling preferentially leads to Th2 responses and TLR4 signaling, on the contrary leads to Th1 development (33). It is therefore particularly interesting that dendritic cells lacking TLR2 or TLR4 signaling following infection with *M. tuberculosis* were still able to imprint Th1 polarity on the naive T cells (Fig. 4), as shown by enhanced production of IFN-γ with concomitant reduction in IL-4 secretion (Fig. 4). These data reiterate that with regard to the interaction of *M. tuberculosis* with dendritic cells, TLR2 and TLR4 are not differentially activated for IL-12 production and subsequently may not contribute differently to the polarization of host adaptive immune responses to the pathogen.



**FIGURE 4.** Lack of either TLR2 or TLR4 does not affect the Th1-polarizing ability of *M. tuberculosis*-infected dendritic cells. BMDCs (1 × 10<sup>6</sup>/ml) from TLR2-deficient or TLR4-defective mice and appropriate wild-type counterparts were either untreated or infected with *M. tuberculosis*. After 24 h, the Transwell (Costar, MA) was placed into 24-well plates containing dendritic cells. Then, naive D011.10 TCR-transgenic T cells (0.5 × 10<sup>6</sup>), irradiated adherent splenocytes (0.3 × 10<sup>6</sup>), and OVA peptide (0.3 μM) were added to the upper chamber. IL-2 was added on day 4. On day 7 poststimulation, T cells were harvested and restimulated with peptide-pulsed irradiated splenocytes in the absence of *M. tuberculosis* (nonbiasing condition). Supernatants were harvested 48 h later and analyzed for the presence of IFN-γ and IL-4 by sandwich ELISA.



**FIGURE 5.** Activation of NF- $\kappa$ B by *M. tuberculosis* in dendritic cells derived from either TLR2-deficient or TLR4-defective mice. A total of  $5 \times 10^6$  BMDCs derived from either TLR2<sup>-/-</sup> mice or from C.C3-Tlr4<sup>Lps-d</sup> mice and appropriate wild-type controls were stimulated with 0.5 mg of irradiated *M. tuberculosis* for 0, 15, 45, and 75 min. Nuclear extracts were prepared and resolved by SDS-PAGE and probed for the presence of the p65 subunit of NF- $\kappa$ B.

#### Absence of TLR2 or TLR4 does not affect *M. tuberculosis*-induced NF- $\kappa$ B nuclear translocation in dendritic cells

Induction of NF- $\kappa$ B translocation is a good indicator of the activation of TLRs, and so we examined nuclear lysates of BMDCs derived from the four strains of mice before and after stimulation with *M. tuberculosis*. In unstimulated BMDCs, a basal level of nuclear translocation of the p65 subunit of NF- $\kappa$ B was observed in all four strains examined, i.e., C57BL/6, TLR2<sup>-/-</sup>, BALB/c, and the TLR4 mutant (Fig. 5). Following stimulation, a substantially increased level of translocation of the p65 subunit was detected in all four groups as early as 15 min (Fig. 5). These data demonstrate that lack of either TLR4 or TLR2 did not affect the activation of the transcription factor NF- $\kappa$ B in BMDCs stimulated with *M. tuberculosis*.

## Discussion

It is well recognized that dendritic cell maturation and cytokine production in response to microbial trigger is critical in modulating host immune responses to the microbe. In the present study, we assessed the contribution of TLR2 and TLR4 to signaling responses in dendritic cells to the pathogen *M. tuberculosis*. Cumulatively, our data indicate that in dendritic cells TLR2 signaling in response to *M. tuberculosis* is qualitatively different from that induced in response to TLR4 and the production of some cytokines is contingent on TLR2 signaling.

The most interesting inference from this study is that production of IL-6 and IL-10, both of which have been implicated in inhibiting macrophage functions and response to IFN- $\gamma$  (34, 35), is dependent on TLR2 signaling. This reveals a novel function for TLR2 and emphasizes the potential importance of this receptor to modulating progression of tuberculosis disease. The IL-10 and IL-6 secretion by TLR2 in response to whole *M. tuberculosis* early on in infection may be part of a negative feedback regulatory mechanism that limits the inflammatory response. However, as disease progresses several *M. tuberculosis* proteins are secreted, and excessive IL-10 and IL-6 secretion through interaction of some of these secreted products with TLR2 may then lead to immunosuppression. In support of this, IL-10-transgenic mice can reactivate a latent tuberculosis infection (36) and TLR2-deficient mice develop an exaggerated immune inflammatory response (37). Furthermore, *Yersinia* virulence factor LcrV can interact with TLR2 to induce IL-10 as an immune evasion strategy that contributes to its virulence (38). It would be of interest to determine whether there are multiple ligands of *M. tuberculosis* for TLR2 and to also study the kinetics of their production and interaction with TLRs on dendritic cells and macrophages as a function of disease progression. A provocative viewpoint is that *M. tuberculosis* in fact usurps the TLR2 system for its own survival. Consistent with this idea, there are studies showing that the 19-kDa lipoprotein of

*M. tuberculosis* can induce macrophage apoptosis (39) and also strongly inhibit macrophage MHC class II expression and Ag processing in a TLR2-dependent fashion (40).

Another conclusion from this study is that in response to *M. tuberculosis*, dendritic cells lacking either TLR2 or TLR4 retain their ability to produce IL-12, a key cytokine in driving Th1 immunity (41). Interestingly, in the absence of TLR2 signaling there is a significant increase in IL-12p40 production from dendritic cells, which may be secondary to their lack of IL-10 production. *M. tuberculosis* heat shock protein 70 can interact with CD40 to produce IL-12 (42, 43), and it is possible that IL-12 production from dendritic cells is mediated via another signaling pathway such as CD40 activation and independent of both TLR2 and TLR4. Another salient observation of this study is that dendritic cell maturation is also independent of TLR2 and TLR4. In addition, release of TNF- $\alpha$  by dendritic cells in response to *M. tuberculosis*, another cytokine that is critical to maintaining host resistance in tuberculosis (44), is also not appreciably hampered in the absence of either TLR2 or TLR4. However, other studies using macrophage cell lines have demonstrated that TNF- $\alpha$  secretion is dependent on both TLR2 and TLR4 signaling. These differences between dendritic cells and macrophages might be due to differences in levels of TLR expression on the two cell types and possibly the presence of specific receptors such as DC-SIGN on the dendritic cells (45, 46). This compensatory mechanism for maturation and IL-12 and TNF- $\alpha$  production in dendritic cells may explain the *in vivo* observations that mice deficient in either TLR2 or TLR4 do not succumb to low-dose *M. tuberculosis* infection.

Using purified ligands of TLR2 and TLR4, it has been shown that TLR2 agonists preferentially induce Th2-promoting cytokines and in contrast downstream events induced by TLR4 ligands are Th1 promoting (33). Our data would suggest that translation of pathogen-derived signals differ significantly when the host cell encounters intact vs soluble products of the pathogen. Intact pathogens possess agonists for more than one TLR protein and intact pathogens may also possess several different agonists for one TLR protein. In this regard, TLR2 can heterodimerize with other TLRs (47) and the different heterodimeric combinations may interact with different ligands on *M. tuberculosis* to activate downstream events that differ and lead to either IL-10 or IL-12 production.

In closing, these studies are particularly important in the context of dendritic cell-based immunization strategies for boosting host resistance against tuberculosis. For example, since IL-10 production is dependent on TLR2, and given the fact that IL-10 can be immunosuppressive, one can design vaccination strategies with dendritic cells lacking TLR2 signaling. The present studies also indicate that it is essential to examine the interaction of the intact pathogen with members of the TLR family since paradigms obtained may differ from those derived from studies using purified ligands.

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## References

1. Medzhitov, R., and C. J. Janeway. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* 10:452.
2. Underhill, D. M., and A. Ozinsky. 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* 14:103.
3. Kaisho, T., and S. Akira. 2000. Critical roles of Toll-like receptors in host defense. *Crit. Rev. Immunol.* 20:393.
4. Hertz, C. J., and R. L. Modlin. 2003. Role of toll-like receptors in response to bacterial infection. *Contrib. Microbiol.* 10:149.
5. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335.

6. Muzio, M., N. Polentarutti, D. Bosisio, P. P. Manoj Kumar, and A. Mantovani. 2000. Toll-like receptor family and signalling pathway. *Biochem. Soc. Trans.* 28:563.
7. Janeway, C. A. J., and R. Medzhitov. 2002. Innate immune recognition. *Annu. Rev. Immunol.* 20:197.
8. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 282:2085.
9. Qureshi, S. T., L. Lariviere, G. Leveque, S. Clermont, K. J. Moore, P. Gros, and D. Malo. 1999. Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). *J. Exp. Med.* 189:615.
10. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.
11. Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. *Science* 284:1313.
12. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782.
13. Sudre, P., G. Ten Dam, and A. Kochi. 1992. Tuberculosis: a global overview of the situation today. *Bull. W.H.O.* 70:149.
14. Flynn, J. L., and J. Chan. 2001. Immunology of tuberculosis. *Annu. Rev. Immunol.* 19:93.
15. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science* 285:732.
16. Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukocyte Biol.* 69:1036.
17. Abel, B., N. Thieblemont, V. J. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl, and B. Ryffel. 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J. Immunol.* 169:3155.
18. Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol.* 163:3920.
19. Means, T. K., E. Lien, A. Yoshimura, S. Wang, D. T. Golenbock, and M. J. Fenton. 1999. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. *J. Immunol.* 163:6748.
20. Means, T. K., B. W. Jones, A. B. Schromm, B. A. Shurtleff, J. A. Smith, J. Keane, D. T. Golenbock, S. N. Vogel, and M. J. Fenton. 2001. Differential effects of a Toll-like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage responses. *J. Immunol.* 166:4074.
21. Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA* 96:14459.
22. Kamath, A. B., J. Alt, H. Debbabi, and S. M. Behar. 2003. Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with *Mycobacterium tuberculosis*. *Infect. Immun.* 71:4112.
23. Reiling, N., C. Holscher, A. Fehrenbach, S. Kroger, C. J. Kirschning, S. Goyert, and S. Ehlers. 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J. Immunol.* 169:3480.
24. Sugawara, I., H. Yamada, C. Li, S. Mizuno, O. Takeuchi, and S. Akira. 2003. Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol. Immunol.* 47:327.
25. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, Ogawa, T., K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11:443.
26. Trizio, D., and G. Cudkovic. 1974. Separation of T and B lymphocytes by nylon wool columns: evaluation of efficacy by functional assays in vivo. *J. Immunol.* 113:1093.
27. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223:77.
28. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 80:707.
29. Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with "mini extracts," prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
30. Hanekom, W. A., M. Mendillo, C. Manca, P. A. Haslett, M. R. Siddiqui, C. Barry III, and G. Kaplan. 2003. Mycobacterium tuberculosis inhibits maturation of human monocyte-derived dendritic cells in vitro. *J. Infect. Dis.* 188:257.
31. Yu, Q., J. X. Gu, C. Kovacs, J. Freedman, E. K. Thomas, and M. A. Ostrowski. 2003. Cooperation of TNF family members CD40 ligand, receptor activator of NF- $\kappa$ B ligand, and TNF- $\alpha$  in the activation of dendritic cells and the expansion of viral specific CD8<sup>+</sup> T cell memory responses in HIV-1-infected and HIV-1-uninfected individuals. *J. Immunol.* 170:1797.
32. Hickman, S. P., J. Chan, and P. Salgame. 2002. *Mycobacterium tuberculosis* induces differential cytokine production from dendritic cells and macrophages with divergent effects on naive T cell polarization. *J. Immunol.* 168:4636.
33. Re, F., and J. L. Strominger. 2001. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. *J. Biol. Chem.* 276:37692.
34. Murray, P. J., L. Wang, C. Onufryk, R. I. Tepper, and R. A. Young. 1997. T cell-derived IL-10 antagonizes macrophage function in mycobacterial infection. *J. Immunol.* 158:315.
35. Nagabhushanam, V., A. Solache, L. M. Ting, C. J. Escaron, J. Y. Zhang, and J. D. Ernst. 2003. Innate inhibition of adaptive immunity: *Mycobacterium tuberculosis*-induced IL-6 inhibits macrophage responses to IFN- $\gamma$ . *J. Immunol.* 171:4750.
36. Turner, J., M. Gonzalez-Juarrero, D. L. Ellis, R. J. Basaraba, A. Kipnis, I. M. Orme, and A. M. Cooper. 2002. In vivo IL-10 production reactivates chronic pulmonary tuberculosis in C57BL/6 mice. *J. Immunol.* 169:6343.
37. Drennan, M. B., D. Nicolle, V. J. Quesniaux, M. Jacobs, N. Allie, J. Mpagi, C. Fremont, H. Wagnet, C. J. Kirschning, and B. Ryffel. 2004. Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. *Am. J. Pathol.* 164:49.
38. Sing, A., D. Rost, N. Tvardovskaia, A. Roggenkamp, A. Wiedemann, C. J. Kirschning, M. Aepfelbacher, and J. Heesemann. 2002. Yersinia V-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med.* 196:017.
39. Lopez, M., L. M. Sly, Y. Luu, D. Young, H. Cooper, and N. E. Reiner. 2003. The 19-kDa *Mycobacterium tuberculosis* protein induces macrophage apoptosis through Toll-like receptor-2. *J. Immunol.* 170:2409.
40. Noss, E. H., R. K. Pai, T. J. Sellati, J. D. Radolf, J. Belisle, D. T. Golenbock, W. H. Boom, and C. V. Harding. 2001. Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. *J. Immunol.* 167:910.
41. Trinchieri, G. 1998. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv. Immunol.* 70:83.
42. Wang, Y., C. G. Kelly, M. Singh, E. G. McGowan, A. S. Carrara, L. A. Bergmeier, and T. Lehner. 2002. Stimulation of Th1-polarizing cytokines, C-C chemokines, maturation of dendritic cells, and adjuvant function by the peptide binding fragment of heat shock protein 70. *J. Immunol.* 169:2422.
43. Lazarevic, V., A. J. Myers, C. A. Scanga, and J. L. Flynn. 2003. CD40, but not CD40L, is required for the optimal priming of T cells and control of aerosol *M. tuberculosis* infection. *Immunity* 19:823.
44. Flynn, J. L., M. M. Goldstein, J. Chan, K. J. Triebold, K. Pfeffer, C. L. Loenstein, R. Schreiber, T. W. Mak, and B. R. Bloom. 1995. Tumor necrosis factor is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity* 2:561.
45. Tailleux, L., O. Schwartz, J. L. Herrmann, E. Pivert, M. Jackson, A. Amara, L. Legres, D. Dreher, L. P. Nicod, J. C. Gluckman, et al. 2003. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. *J. Exp. Med.* 197:121.
46. Geijtenbeek, T. B., S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vandenbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk. 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* 197:7.
47. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA* 97:13766.