Codominance of TLR2-Dependent and TLR2-Independent Modulation of MHC Class II in *Mycobacterium tuberculosis* Infection In Vivo

Eleanor Z. Kincaid, Andrea J. Wolf, Ludovic Desvignes, Sebabrata Mahapatra, Dean C. Crick, Patrick J. Brennan, Martin S. Pavelka, Jr. and Joel D. Ernst

*J Immunol* 2007; 179:3187-3195; doi: 10.4049/jimmunol.179.5.3187

http://www.jimmunol.org/content/179/5/3187

---

**References**  This article cites 57 articles, 43 of which you can access for free at: http://www.jimmunol.org/content/179/5/3187.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Codominance of TLR2-Dependent and TLR2-Independent Modulation of MHC Class II in Mycobacterium tuberculosis Infection In Vivo

Eleanor Z. Kincaid,*† Andrea J. Wolf,*† Ludovic Desvignes,* Sebabrata Mahapatra,‡ Dean C. Crick,§ Patrick J. Brennan,‡ Martin S. Pavelka, Jr.,∥ and Joel D. Ernst2*†∥

Mycobacterium tuberculosis is an exceptionally successful human pathogen. A major component of this success is the ability of the bacteria to infect immunocompetent individuals and to evade eradication by an adaptive immune response that includes production of the macrophage-activating cytokine, IFN-γ. Although IFN-γ is essential for arrest of progressive tuberculosis, it is insufficient for efficacious macrophage killing of the bacteria, which may be due to the ability of M. tuberculosis to inhibit selected macrophage responses to IFN-γ. In vitro studies have determined that mycobacterial lipoproteins and other components of the M. tuberculosis cell envelope, acting as agonists for TLR2, inhibit IFN-γ induction of MHC class II. In addition, M. tuberculosis peptidoglycan and IL-6 secreted by infected macrophages inhibit IFN-γ induction of MHC class II in a TLR2-independent manner. To determine whether TLR2-dependent inhibition of macrophage responses to IFN-γ is quantitatively dominant over the TLR2-independent mechanisms in vivo, we prepared mixed bone marrow chimeric mice in which the hemopoietic compartment was reconstituted with a mixture of TLR2+/+ and TLR2−/− cells. When the chimeric mice were infected with M. tuberculosis, the expression of MHC class II on TLR2+/+ and TLR2−/− macrophages from the lungs of individual infected chimeric mice was indistinguishable. These results indicate that TLR2-dependent and -independent mechanisms of inhibition of responses to IFN-γ are equivalent in vivo, and that M. tuberculosis uses multiple pathways to abrogate the action of an important effector of adaptive immunity.

Received for publication April 4, 2007. Accepted for publication June 22, 2007.

Lifelong persistence is a hallmark of tuberculosis infection (1), despite development of a vigorous Th1 immune response in the majority of infected humans (2). Mouse models of infection have recapitulated this observation, and have demonstrated that the appearance of Mycobacterium tuberculosis Ag-specific CD4+ T cells in the lungs coincides with control of bacterial growth (3) and established that IFN-γ is essential for arresting progressive infection (4, 5). Although IFN-γ plays an essential role in the control of M. tuberculosis infection, its induction and secretion are not sufficient to achieve sterile immunity: M. tuberculosis persists despite high concentrations of IFN-γ at sites of infection (6–8). The observation that IFN-γ activates macrophages to kill intracellular pathogens such as Leishmania (9, 10), Toxoplasma (11–13), and Legionella (14, 15), but is incapable of activating macrophages to kill M. tuberculosis unless IFN-γ stimulation precedes infection (9, 16, 17), led to the hypothesis that M. tuberculosis may interfere with macrophage responses to IFN-γ to avoid elimination by the adaptive immune response. Indeed, experiments using in vitro infection of human and murine macrophages have shown that whole M. tuberculosis and specific subcellular components of the bacteria inhibit macrophage responses to IFN-γ, resulting in decreased induction of MHC class II and Ag presentation to CD4+ T cells (18–24), as well as decreased induction of NO-independent antimicrobial activity (13, 19, 25). Among the subcellular components of M. tuberculosis that contribute to inhibition of responses to IFN-γ are several bacterial lipoproteins, including a 19-kDa lipoprotein, LprA, and LprG (19–21, 24, 26, 27), and the cell wall lipoglycan phosphatidylinositol mannan (18), all of which exert their effects by serving as agonists of TLR2. In support of a potential role for TLR2 signaling in inhibiting cellular responses to IFN-γ in vivo is the observation that, after low-dose aerosol infection with M. tuberculosis, TLR2−/− mice expressed higher levels of MHC class II on the surface of CD11c+ and Ly6G+ lung cells, but interpretation of that result is confounded by the observation that those mice also expressed higher levels of IFN-γ in their lungs (28). When infected with higher inocula, TLR2−/− mice controlled M. tuberculosis infection less well than wild-type controls, with higher bacterial burdens in the lungs and shorter survival (28–30). Because TLR2 agonist treatment in vitro results in induction of proinflammatory cytokine secretion and dendritic cell (DC) maturation (31, 32) as well as inhibition of macrophage responses to IFN-γ (18–21, 27),

*Division of Infectious Diseases, Department of Medicine, New York University School of Medicine, New York, NY 10016; †Biomedical Sciences Graduate Program, University of California, San Francisco, CA 94143; ‡Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523; §Department of Microbiology and Immunology, University of Rochester, Rochester, NY 14642; and Departments of Pathology and Microbiology, New York University School of Medicine, New York, NY 10016

Received for publication April 4, 2007. Accepted for publication June 22, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by National Institutes of Health Grants AB04697, AB051242, AB059667 (to J.D.E.), AI47311 (to M.S.P.), AB065357-020010, AB049151 (to D.C.C.), and AI018357 (to P.J.B.); a grant from the College Research Council, College of Veterinary Medicine and Biomedical Sciences, Colorado State University; and a graduate student fellowship from the Western Affiliate of the American Heart Association (to E.Z.K.).

Address correspondence and reprint requests to Dr. Joel D. Ernst, New York University School of Medicine, Smilow Research Center, Room 901, 550 First Avenue, New York, NY 10016. E-mail address: joel.ernst@med.nyu.edu

www.jimmunol.org

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00
the overall susceptibility of TLR2−/− mice is likely to be the net result of a complex interplay between these responses.

In addition to the TLR2-dependent inhibition of macrophage responses to IFN-γ, we have also found evidence for TLR2-independent mechanisms of inhibition. When we compared the activity of a M. tuberculosis lysate to inhibit induction of the IFN-γ-responsive gene, CIITA, in TLR2+/+ and TLR2−/− bone marrow-derived macrophages (BMDM), we found that TLR2−/− macrophages were 100-fold less sensitive to the effects of this M. tuberculosis lysate than were TLR2+/+ macrophages. When we tested the sensitivity of TLR2+/+ and TLR2−/− macrophages to inhibition by live M. tuberculosis, we found that, although there was still a difference in the sensitivity to inhibition by M. tuberculosis, it was smaller (a 16-fold difference), suggesting that the process of homogenization increased the availability of M. tuberculosis TLR2 agonists, because the whole cell lysates also activated a TLR2 reporter cell line much more potently than intact bacteria. Therefore, it is possible that the effects of TLR2 agonists are overemphasized by experiments with M. tuberculosis whole cell lysates or isolated agonists. These results indicate that a TLR2-dependent mechanism is dominant in vitro, but that one or more TLR2-independent mechanisms clearly contribute to inhibition of macrophage responses to IFN-γ. A candidate for this TLR2-independent activity in whole M. tuberculosis is the mycolyl-arabinogalactan-peptidoglycan complex of the M. tuberculosis cell wall, which we have previously found to inhibit IFN-γ induction of CIITA by a TLR2- and MyD88-independent mechanism. This was still a difference in the sensitivity to inhibition by M. tuberculosis macrophage responses to IFN-γ, and TLR2-independent mechanisms clearly contribute to inhibition of M. tuberculosis by purified mycolic acids or arabinogalactan (18, 19).

In addition to the TLR2-dependent inhibition of macrophage activity in whole M. tuberculosis, we have also found evidence for TLR2-independent activity in vitro, which is most likely derived from the peptidoglycan itself (35), indicating a highly pure peptidoglycan preparation. The purified peptidoglycan was solubilized by digestion with purified Chalaraopsis spp. muramidase and M. smegmatis peptidoglycan, filtered through a 10-kDa ultrafiltration membrane to remove muramidase, and transferred to a preweighed tube and dried under vacuum for quantitation.

Preparation of Escherichia coli peptidoglycan
E. coli K12 (American Type Culture Collection No. 19215) was grown in Luria broth (EM Science) to an OD600 of 1.0. The cells were harvested and washed with PBS, and peptidoglycan was prepared, as described (37). The purity of the peptidoglycan was checked by amino acid analysis, and the muropeptides were solubilized using Chalaraopsis muramidase treatment, as described above.

Cell culture
Bone marrow cells were isolated and cultured, as previously described (18, 38). One day before treatment with mycobacteria, BMDM were replated at 5 × 104/well in 12-well tissue culture plates in DMEM supplemented with 10% FCS, 10% L929 cell-conditioned medium, 1 μM sodium pyruvate, and 2 mM t-glutamine. RAW264.7 cells (American Type Culture Collection) were grown in DMEM with 10% heat-inactivated FBS and 2 mM t-glutamine (all obtained from Invitrogen Life Technologies). Cells were allowed to adhere overnight before treatment with bacterial components. Cells were treated with gamma-irradiated M. tuberculosis or peptidoglycan for 8–24 h, followed by overnight treatment with 20 ng/ml murine IFN-γ (BD Pharmingen).

Preparation of bone marrow
Bone marrow cells were isolated from C57BL/6CD45.1 and TLR2−/− mice, as previously described (38). The cells were subjected to ACK lysis to remove erythrocytes, rinsed with DMEM, and stored briefly on ice in a 1:1 mixture of bone marrow cells from 6- to 7-wk-old female CD45.1 and TLR2−/− bone marrow. We reasoned that by analyzing TLR2+/+ and TLR2−/− cells isolated from the same infected mouse, we would obviate the potential effects of variables such as the bacterial load and concentrations of stimulatory and inhibitory cytokines that may exist between TLR2+/+ and TLR2−/− mice. We infected these mixed bone marrow chimeras with M. tuberculosis by the aerosol route, and analyzed MHC class II expression on alveolar macrophages, DC, and recruited macrophages from the lungs. We found that the absence of TLR2 did not alter the surface expression of MHC class II on alveolar macrophages, recruited macrophages, or DC from the lungs of infected mice at 21, 28, or 35 days postinfection. These results suggest that TLR2-dependent and -independent mechanisms are involved both in inhibition of macrophage responses to IFN-γ and in maturation of DC during M. tuberculosis infection in vivo.

Materials and Methods

Mice
C57BL/6, C57BL/6 congenic CD45.1, IFN-γR−/−, and TLR2−/− mice were obtained from The Jackson Laboratory and bred and maintained under specific pathogen-free conditions. IFN-γR−/− and TLR2−/− mice from The Jackson Laboratory have been backcrossed onto the C57BL/6 background for 10 or 9 generations, respectively. Infected mice were housed under barrier conditions in an Animal Biosafety Level 3 facility. All work with animals was approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Preparation and solubilization of M. tuberculosis and Mycobacterium smegmatis peptidoglycan
The gamma-irradiated M. tuberculosis H37Rv (Colorado State University, National Institutes of Health, National Institute of Allergy and Infectious Diseases Contract N01 AI-75520) was prepared, as described previously (22). To prepare mycobacterial peptidoglycan, M. tuberculosis H37Rv or M. smegmatis was grown in glycerol-alanine-salt medium. Midlog phase cells were harvested by centrifugation, washed, and sterilized by gamma irradiation. Irradiated cells were suspended in PBS and disrupted by probe sonication on ice, followed by digestion with DNase and RNase, and a cell wall-anchored pellet was obtained by differential centrifugation. Peptidoglycan was prepared from the cell wall fraction essentially as previously reported (33, 34) with minor modifications, and the purity of the preparation was verified by amino acid analysis after acid hydrolysis. Results showed the presence of alanine, glutamic acid, and diaminopimelic acid with virtually undetectable levels of a small number of other amino acids, which were most likely derived from the peptidoglycan itself (35), indicating a highly pure peptidoglycan preparation. The purified peptidoglycan was solubilized by digestion with purified Chalaraopsis spp. muramidase and M. smegmatis peptidoglycan, filtered through a 10-kDa ultrafiltration membrane to remove muramidase, and transferred to a preweighed tube and dried under vacuum for quantitation.

Preparation of Escherichia coli peptidoglycan
E. coli K12 (American Type Culture Collection No. 19215) was grown in Luria broth (EM Science) to an OD600 of 1.0. The cells were harvested and washed with PBS, and peptidoglycan was prepared, as described (37). The purity of the peptidoglycan was checked by amino acid analysis, and the muropeptides were solubilized using Chalaraopsis muramidase treatment, as described above.

Cell culture
Bone marrow cells were isolated and cultured, as previously described (18, 38). One day before treatment with mycobacteria, BMDM were replated at 5 × 104/well in 12-well tissue culture plates in DMEM supplemented with 10% FCS, 10% L929 cell-conditioned medium, 1 μM sodium pyruvate, and 2 mM t-glutamine. RAW264.7 cells (American Type Culture Collection) were grown in DMEM with 10% heat-inactivated FBS and 2 mM t-glutamine (all obtained from Invitrogen Life Technologies). Cells were allowed to adhere overnight before treatment with bacterial components. Cells were treated with gamma-irradiated M. tuberculosis or peptidoglycan for 8–24 h, followed by overnight treatment with 20 ng/ml murine IFN-γ (BD Pharmingen).

Preparation of bone marrow
Bone marrow cells were isolated from C57BL/6CD45.1 and TLR2−/− mice, as previously described (38). The cells were subjected to ACK lysis to remove erythrocytes, rinsed with DMEM, and stored briefly on ice in a 1:1 mixture of bone marrow cells from 6- to 7-wk-old female CD45.1 and TLR2−/− bone marrow. We reasoned that by analyzing TLR2+/+ and TLR2−/− cells isolated from the same infected mouse, we would obviate the potential effects of variables such as the bacterial load and concentrations of stimulatory and inhibitory cytokines that may exist between TLR2+/+ and TLR2−/− mice. We infected these mixed bone marrow chimeras with M. tuberculosis by the aerosol route, and analyzed MHC class II expression on alveolar macrophages, DC, and recruited macrophages from the lungs. We found that the absence of TLR2 did not alter the surface expression of MHC class II on alveolar macrophages, recruited macrophages, or DC from the lungs of infected mice at 21, 28, or 35 days postinfection. These results suggest that TLR2-dependent and -independent mechanisms are involved both in inhibition of macrophage responses to IFN-γ and in maturation of DC during M. tuberculosis infection in vivo.
infected with a strain of *M. tuberculosis* H37Rv that constitutively expresses GFP (57) at a dose of 231 (±100) bacteria per mouse. In addition, six C57BL/6 mice were infected with *M. tuberculosis* H37Rv at a dose of 234 (±81) bacteria per mouse. Lung cells from these H37Rv-infected mice were used for flow cytometry to set the boundary between GFP-positive and -negative populations and as compensation controls, as previously described (57). For each infection, the effective bacterial intake was determined by euthanizing five control mice within 24 h of exposure, and assaying the bacterial load in their lungs, as previously described (57).

**Tissue harvest and bacterial load determination**

Mice were euthanized by CO2 narcosis, followed by cervical dislocation. The lungs were excised, minced, and subjected to enzymatic digestion, as previously described (39). An aliquot of this lung homogenate was stored at −80 °C for later CFU counts. The single-cell suspension was washed, and erythrocytes were removed by hypotonic lysis, as previously described (57). Live cells were counted using a hemacytometer and trypan blue exclusion.

**Flow cytometry**

In vitro samples were prepared, as previously described (18), and stained with PE-conjugated anti-I-A/I-E (clone M5/114.15.2; BD Biosciences). For analysis of samples isolated from infected mice, lung cells were incubated in FcR blocking Ab (2.4G2 hybridoma supernatant) for 10 min, followed by a 20-min incubation with the indicated Abs. For the IFN-γ–K−− experiment, the following were used: FITC-conjugated anti-I-A/I-E (clone M5/114.15.2; BD Biosciences), PE-conjugated anti-CD11c (clone HL3; BD Biosciences), and allophycocyanin-conjugated CD11b (clone M1/70; BD Biosciences). To quantitate chimerism, the following were used: PE-conjugated anti-CD45.1 (clone A20; BD Biosciences), Pacific Blue-conjugated anti-CD45.2 (clone 104; BioLegend), allophycocyanin-Cy7-conjugated anti-CD11b (clone M1/70; BD Biosciences), and PerCP-conjugated anti-CD11c (clone HL3; custom conjugate from BD Biosciences). To determine MHC class II expression in the mixed bone marrow chimeras, the following were used: I-A/I-E (clone M5/114.15.2; purified Ab from BD Biosciences conjugated using Molecular Probes AlexaFluor647 protein-labeling kit) and Pacific Blue-conjugated anti-CD45.2, allophycocyanin-Cy7-conjugated anti-CD11b, and PerCP-conjugated anti-CD11c (as described above). Cells were then washed with staining buffer (Dulbecco’s PBS, 1% FCS, 0.1% sodium azide, and 1 mM EDTA) and fixed in 1% paraformaldehyde in Dulbecco’s PBS overnight at 4 °C. Samples were acquired using a FACSComp aur or an LSR II flow cytometer, depending on the experiment (both machines from BD Biosciences). Data were acquired with CellQuest software (BD Biosciences) and analyzed with FlowJo software (Tree Star). Lung myeloid DC, alveolar macrophages, recruited macrophages, and GFP-positive *M. tuberculosis*-infected cells were identified by their levels of expression of CD11c and CD11b, as previously described (57).

**Statistical analysis**

Prism 4.0 (GraphPad) was used for all statistical analysis, which included Wilcoxon matched pairs test and two-way ANOVA.

**Results**

**TLR2−/− macrophages are less susceptible to *M. tuberculosis* inhibition of responses to IFN-γ in vitro**

We have previously found that TLR2−/− BMDM are relatively resistant to inhibition of responses to IFN-γ by *M. tuberculosis* compared with TLR2+/+ BMDM. This difference in sensitivity is found when cells are infected with live *M. tuberculosis* or treated with a *M. tuberculosis* whole cell lysate, but the difference is quantitatively greater when cells are treated with the *M. tuberculosis* whole cell lysate (18). To better understand the difference between live and homogenized *M. tuberculosis*, we examined a whole, inactivated form of *M. tuberculosis*. We treated bone marrow macrophages from TLR2+/+ and TLR2−/− mice with whole, gamma-irradiated *M. tuberculosis*, followed by IFN-γ, and assayed surface expression of MHC class II (Fig. 1). We found that, whereas half-maximal inhibition of TLR2+/+ macrophages was attained with 29 μg/ml gamma-irradiated *M. tuberculosis*, 132 μg/ml was required for the same level of inhibition in TLR2−/− macrophages. Together, our findings suggest that *M. tuberculosis* TLR2 agonists are very potent in soluble form, but that insoluble or otherwise sequestered components of *M. tuberculosis* make a significant contribution to inhibition of macrophage responses to IFN-γ by intact *M. tuberculosis* by TLR2-dependent and TLR2-independent mechanisms.

**Purified *M. tuberculosis* peptidoglycan is more potent than *E. coli* peptidoglycan for induction of IFN-γ induction of MHC class II**

We have previously found that mycolyl-arabinogalactan-peptidoglycan, the detergent-insoluble, protease-resistant fraction of the *M. tuberculosis* cell wall, inhibits macrophage responses to IFN-γ in TLR2−/− BMDM, and that peptidoglycan is the active component of this fraction. These findings raised the question whether *M. tuberculosis* peptidoglycan differs from other bacterial peptidoglycans in this property. To answer this question, we compared the potency of purified *M. tuberculosis* peptidoglycan with that of purified *E. coli* peptidoglycan (Fig. 2). Although pretreatment of macrophages with *E. coli* peptidoglycan inhibited IFN-γ induction of surface MHC class II by ~25% at a concentration of 10 μg/ml, *M. tuberculosis* peptidoglycan caused this extent of inhibition at a lower concentration (0.5 μg/ml), and caused ~95% inhibition at a...
mice that require IFN-γ dependence of MHC class II expression on alveolar macrophages, recruited macrophages, and myeloid DC from the lungs of M. tuberculosis-infected mice. MHC class II expression on alveolar macrophages (A), recruited macrophages (B), and myeloid DC (C), from the lungs of representative C57BL/6 (tiled histograms) and IFN-γR−/− (open histograms) mice 28 days postinfection. The experiment included five C57BL/6 mice and four IFN-γR−/− mice.

concentration of 10 μg/ml. Therefore, M. tuberculosis peptidoglycan is more potent at inhibiting macrophage responses to IFN-γ, and suggests that a structural difference accounts for this difference. One known structural difference in mycobacterial peptidoglycans is that a high proportion of muramic acid subunits is N-glycolylated, rather than N-acetylated as in other bacteria (40). To determine whether this structural modification accounts for the greater potency of M. tuberculosis peptidoglycan, we compared the activities of peptidoglycan purified from wild-type M. smegmatis or from a mutant strain of M. smegmatis lacking the enzyme, UDP-N-acetylmuramic acid hydroxylase, which is essential for glycolylation of muramic acids in mycobacteria (41). Peptidoglycans from wild-type and UDP-N-acetylmuramic acid hydroxylase mutant M. smegmatis were indistinguishable in their potency for inhibiting macrophage responses to IFN-γ (data not shown), indicating that this modification does not account for the difference observed between mycobacterial and E. coli peptidoglycans.

IFN-γ dependence of MHC class II surface expression on lung macrophage subsets and DC in vivo

Although TLR2-dependent mechanisms of inhibition are dominant in vitro, the balance may be altered in the in vivo environment, where the kinetics of infection are less synchronous and more dynamic, and new cells are recruited during the course of infection. To determine the role of TLR2 in modulating macrophage responses to IFN-γ in the lungs of mice infected with M. tuberculosis, we first characterized the cells from the lungs of infected mice that require IFN-γ responsiveness for MHC class II expression. We infected wild-type and IFN-γR knockout (IFN-γR−/−) mice with M. tuberculosis, and assessed MHC class II expression on the predominant subsets of myeloid cells in the lungs of M. tuberculosis-infected mice after 28 days of infection. Using a previously validated set of gates (42–44, 57), we identified CD11chighCD11blow alveolar macrophages, CD11clowCD11bmedium recruited macrophages, and CD11chighCD11bhigh lung myeloid DC. We found that surface expression of MHC class II on alveolar and recruited macrophages depended on responsiveness to IFN-γ. Although a subset of IFN-γR−/− alveolar macrophages expressed high levels of MHC class II, most expressed ~10-fold lower levels compared with that of wild-type mice (Fig. 3A). In IFN-γR−/− mice, recruited macrophages expressed only low to intermediate levels of MHC class II compared with alveolar macrophages from the same mouse. The intermediate level of MHC class II expression was not found on recruited macrophages from the lungs of IFN-γR−/− mice; these cells were uniformly MHC class II+ (Fig. 3B). This observation provides evidence that the gate settings used to identify recruited macrophages effectively excluded the subset of alveolar macrophages that expressed high levels of surface MHC class II in an IFN-γ-independent manner. Surface expression of MHC class II on myeloid DC from the lungs of M. tuberculosis-infected IFN-γR−/− mice reached peak levels equivalent to those on cells of wild-type C57BL/6 mice (Fig. 3C). This finding is consistent with the observation that expression of high levels of surface MHC class II on DC accompanies their maturation and occurs independently of IFN-γ in vitro (45). These results indicate that analysis of expression of surface MHC class II can be used as an indicator of responsiveness to IFN-γ, especially on recruited macrophages isolated from the lungs.

TLR2 mixed bone marrow chimeras are competent for short-term control of M. tuberculosis infection

To compare the sensitivity of TLR2+/+ and TLR2−/− macrophages with inhibition by M. tuberculosis in vivo, we prepared mixed bone marrow chimeras with bone marrow from TLR2+/+ and TLR2−/− mice. We exposed TLR2+/+ CD45.1 mice to a lethal dose of irradiation and reconstituted their bone marrow with a 1:1 mixture of bone marrow from TLR2+/+ CD45.1 and TLR2−/− CD45.2 mice (Fig. 4A). These mice were then allowed to recover for 8 wk before aerosol infection with ~230 M. tuberculosis bacilli per mouse. At day 18 postinfection, we tested the chimerism of the cells in the lungs. We found that both TLR2+/+ CD45.1 and TLR2−/− CD45.2 cells were represented in the lung, but that there were slightly more CD45.1 (TLR2+/+) than CD45.2 (TLR2−/−) cells (Fig. 4B). This trend was observed throughout the course of the experiment (Fig. 4C) (p < 0.01, by two-way ANOVA). At days 18, 21, 28, and 35 postinfection, we quantitated the bacterial loads in the lungs. We found that, consistent with the findings in nonchimeric TLR2+/+ and TLR2−/− mice (28–30), the mixed bone marrow chimeric mice were able to arrest progression of bacterial replication in the lung between days 21 and 35 (Fig. 5). In addition, none of the chimeric mice became moribund during the course of the experiment.

MHC class II expression is not higher on macrophages lacking TLR2

Alveolar macrophages are believed to be the first cells to encounter M. tuberculosis after aerosol infection. Using flow cytometry to simultaneously identify the CD11chighCD11blow alveolar macrophage population (46) and GFP+ M. tuberculosis-infected cells, we have detected M. tuberculosis-infected alveolar macrophages as early as 14 days after aerosol infection (A. Wolf, B. Linas, E. Kincaid, T. Tamura, K. Takatsu, and J. Ernst, submitted for publication). The number of alveolar macrophages, however, does not increase during the course of infection in mice, so the relative contribution of this cell type to the total bacterial load decreases as the number of infected DC and recruited macrophages increases. In vitro, IFN-γ treatment induces increased MHC class II expression on alveolar macrophages, and TLR2 agonist treatment inhibits this induction (26). In vivo, expression of MHC class II on alveolar macrophages is largely, but not exclusively, dependent on IFN-γ (Fig. 3A).

To examine the role of TLR2 in MHC class II expression on alveolar macrophages during M. tuberculosis infection, we compared MHC class II expression on TLR2+/+ and TLR2−/− alveolar macrophages. We focused our analysis on times after the onset of adaptive immunity, when significant amounts of IFN-γ are expressed in the lung (47, 48). We tested the chimerism of the alveolar macrophage population in mixed bone marrow recipient mice and we found that both TLR2+/+ (CD45.2) and TLR2−/− (CD45.1) cells were represented in this leukocyte subset (data not shown).
shown). We found no significant difference in MHC class II expression between TLR2\(^{-/-}\) and TLR2\(^{+/+}\) alveolar macrophages on day 21, 28, or 35 postinfection (by Wilcoxon matched pairs test) (Fig. 6, A–C).

We also examined surface expression of MHC class II on CD11clowCD11bmedium recruited macrophages, which increase 5-fold in number in the lungs between days 14 and 35 of infection and represent the second largest population of \(M.\) tuberculosis-infected cells in the lungs (A. Wolf, B. Linas, E. Kincaid, T. Tamura, K. Takatsu, and J. Ernst, submitted for publication). Compared with alveolar macrophages, recruited macrophages express lower levels of MHC class II during \(M.\) tuberculosis infection (A. Wolf, B. Linas, E. Kincaid, T. Tamura, K. Takatsu, and J. Ernst, submitted for publication), but like cultured macrophages, their MHC class II expression is dependent on IFN-\(\gamma\) (Fig. 3). We found that both TLR2\(^{-/-}\) (CD45.2) and TLR2\(^{+/+}\) (CD45.1) cells were represented in the recruited macrophage population (Fig. 4, D and E). We found no significant difference in the surface MHC class II expression on TLR2\(^{-/-}\) or TLR2\(^{+/+}\) cells from the lungs of infected mice, harvested 21, 28, or 35 days after \(M.\) tuberculosis infection (Fig. 6, D–F).

FIGURE 5. Mixed bone marrow chimeric mice control \(M.\) tuberculosis infection. Lungs of mixed bone marrow chimeras infected with \(M.\) tuberculosis H37Rv-GFP were harvested at 18, 21, 28, and 35 days postinfection, and homogenates were plated on Middlebrook 7H11 agar. Day 1 CFUs are from control CD45.1 mice, and are included for reference.

FIGURE 6. MHC class II expression on alveolar macrophages. At 21, 28, and 35 days postinfection, lungs of \(M.\) tuberculosis-infected bone marrow chimeric mice were harvested and single-cell suspensions were analyzed by flow cytometry. Cells were gated for alveolar macrophages or recruited macrophages, based on their levels of expression of surface CD11c and CD11b. MHC class II expression on TLR2\(^{-/-}\) (CD45.2-negative, \(\bullet\)) and TLR2\(^{+/+}\) (CD45.2-positive, \(\bigcirc\)) cells from the same mouse was compared. There was no significant difference in MHC class II expression on TLR2\(^{-/-}\) (CD45.2-negative, \(\bullet\)) and TLR2\(^{+/+}\) (CD45.2-positive, \(\bigcirc\)) alveolar macrophages at 21 (A), 28 (B), or 35 (C) days postinfection. There was also no significant difference between MHC class II expression on TLR2\(^{-/-}\) (CD45.2-negative, \(\bullet\)) and TLR2\(^{+/+}\) (CD45.2-positive, \(\bigcirc\)) recruited macrophages at 21 (D), 28 (E), or 35 (F) days postinfection. Each pair of dots connected by a line represents the results from an individual mouse. The geometric means of MHC class II expression in each group were compared by Wilcoxon matched pairs test.
TLR2 expression does not impact MHC class II surface expression on recruited macrophages that contain M. tuberculosis

In cultured macrophages, we have found that infected cells as well as uninfected cells in the same well show decreased responsiveness to IFN-γ, and that conditioned medium from infected cells can reproduce this inhibition in naive cells (38). The presence of one or more soluble mediators suggests that host responses to M. tuberculosis contribute to the inhibition of macrophage MHC class II expression, or that bacterial components released from infected macrophages may exert effects on bystander cells. However, it is unclear whether a similar effect occurs in the environment of infected lungs, and the relative contribution of pathogen-derived and host-derived factors in macrophage inhibition in vivo is unknown. To investigate the impact of M. tuberculosis-derived TLR2 agonists on MHC class II expression on M. tuberculosis-infected cells in vivo, we compared MHC class II expression on TLR2+/+ and TLR2−/− recruited macrophages that contain bacteria and those that do not contain bacteria, after isolation from lungs of infected mice. We have previously validated the use of GFP-expressing M. tuberculosis H37Rv to identify infected cells from single-cell suspensions of whole infected lungs (A. Wolf, B. Linas, E. Kincaid, T. Tamura, K. Takatsu, and J. Ernst, submitted for publication), and we used this technique to identify infected recruited macrophages and quantitate their MHC class II expression. We found that at 21, 28, and 35 days postinfection, there was no significant difference between the MHC class II expression of M. tuberculosis-infected TLR2+/+ and TLR2−/− recruited macrophages (Fig. 7, A–C). This indicates that in the subset of infected cells, as well as in the population of recruited macrophages as a whole, the presence or absence of TLR2 does not have a quantitative influence on the expression of IFN-γ-dependent surface MHC class II. Because isolating this population involves sequential gating analysis, the number of events in the TLR2+/+ or TLR2−/− GFP-positive, recruited macrophage population in some animals was small. All animals were included in the data analysis, however, because samples with <300 events followed the same trend as those with >300 events. The number of GFP+ alveolar macrophages was not sufficient to allow statistically valid analysis of MHC class II expression on M. tuberculosis-infected alveolar macrophages.

We also investigated whether M. tuberculosis-infected cells exhibit differential expression of MHC class II compared with the whole population of recruited macrophages, irrespective of TLR2 expression. We compared surface MHC class II expression on infected and uninfected recruited macrophages, and we found that although there was no significant difference in the MHC class II expression between these two populations at any time point, there was a trend toward higher MHC class II expression on infected macrophages over the course of the experiment (Fig. 7, D–F). At day 21 postinfection, five of six mice had lower MHC class II expression on infected vs uninfected recruited macrophages, but by day 35 postinfection the pattern was reversed. The statistically equivalent MHC class II expression on the two populations suggests that M. tuberculosis establishes an inhibitory environment in the lungs that affects both infected and uninfected macrophages.

TLR2 is not required for high MHC II expression on DC in lungs of M. tuberculosis-infected mice

Although MHC class II expression in macrophages depends on IFN-γ-responsive CIITA expression, MHC class II surface expression in DC is part of a regulated maturation process induced by pathogen-associated molecules and proinflammatory cytokines. During this process, translation of new MHC class II molecules is stopped and the MHC class II molecules already present in the cell are loaded with Ag and trafficked to the cell surface. The mature, MHC class IIhigh DC are efficient APCs with the potential to prime naive T lymphocytes.

Infection with live M. tuberculosis or treatment with mycobacterial TLR2 agonists leads to DC maturation in vitro (32, 42, 49, 50). However, regulation of DC maturation in vivo during M. tuberculosis infection is likely to be a complex interplay of pathogen and host-derived proinflammatory stimuli. To investigate the role of TLR2 in DC MHC class II surface expression during M. tuberculosis infection, we focused on a population of CD11chighCD11bhigh cells that we and others have identified as lung myeloid DC (42–44) (A. Wolf, B. Linas, E. Kincaid, T. Tamura, K. Takatsu, and J. Ernst, submitted for publication). As with the macrophage populations, we found that both TLR2+/+ (CD45.2) and TLR2−/− (CD45.1) cells were represented in this leukocyte subset (data not shown). We found that at 21, 28, and 35 days postinfection, there was no significant difference between the MHC class II expression of TLR2+/+ and TLR2−/− DC (Fig. 8, A–C). We also found no effect of TLR2 on the MHC class II expression of M. tuberculosis-infected (GFP+) DC on day 21, 28, or 35 (Fig. 8, D–F).

We compared MHC class II expression on uninfected and infected DC, and we found that at 21 days postinfection, MHC class II expression on M. tuberculosis-infected DC was slightly, but significantly higher than on uninfected DC (Fig. 8G, p < 0.05).
contrast, there was no significant difference between the two populations at 28 or 35 days postinfection (Fig. 8, H and I). These results indicate that early in infection, direct infection of DC contributes to their maturation, but that later in infection, infected and uninfected DC exhibit equivalent maturation, most likely due to the induction and action of proinflammatory cytokines such as TNF.

Discussion

*M. tuberculosis* has evolved efficacious mechanisms for evading innate and adaptive immunity, as indicated by the massive number of humans it has infected, and by the observation that the immune responses of humans and experimental animals are unable to eradicate the infection. Despite the importance of immune evasion in the pathogenesis of tuberculosis, its mechanisms are still incompletely understood. One of the most-studied mechanisms of *M. tuberculosis* evasion of adaptive immunity is its inhibition of selected responses to IFN-γ in cultured macrophages (13, 18–22, 24, 26, 27, 51). Although *M. tuberculosis* does not inhibit induction of all IFN-γ-responsive genes in cultured macrophages (i.e., inducible NO synthase is not affected (19, 25)), inhibition of induction of MHC class II, due to inhibition of induction of CIITA, is among the quantitatively most profoundly affected responses in vitro. Because a TLR2-dependent mechanism of *M. tuberculosis* inhibition of IFN-γ-dependent macrophage expression of MHC class II has been well characterized, we tested the hypothesis that this mechanism is dominant in vivo. If this were the case, we predicted that we would find higher expression of MHC class II on TLR2-deficient macrophages than on TLR2-replete macrophages when isolated from *M. tuberculosis*-infected mice that contained macrophages of both phenotypes in their lungs. However, we found that TLR2-deficient and TLR2-replete recruited lung macrophages express indistinguishable levels of surface MHC class II during *M. tuberculosis* infection in vivo. This finding is compatible with several interpretations. First, although lipoproteins and other TLR2 agonists are dominant in short-term in vitro assays, the TLR2-independent *M. tuberculosis* component(s) may have an equivalent inhibitory effect at later times after a cell is infected in vivo. We have found that *M. tuberculosis* TLR2 agonists are more potent in a soluble form than when presented as part of intact mycobacteria. This finding suggests that TLR2 agonists may act predominantly when the *M. tuberculosis* bacilli are extracellular or early after infection. In contrast, *M. tuberculosis* peptidoglycan is not likely to be exposed on the surface of the bacteria, and may therefore act after the bacteria have been internalized or after peptidoglycan is shed by bacteria replicating inside macrophages. Our results are also consistent with the interpretation that TLR2-independent mechanisms of inhibition of IFN-γ-dependent class II expression are actually dominant over the TLR2-dependent mechanisms in vivo. Although this is formally possible, the strong evidence for TLR2-dependent mechanisms identified in vitro studies makes it more likely that the mechanisms are codominant or equipotent in vivo.

A second potential interpretation of the observation that TLR2-deficient and TLR2-replete recruited macrophages express the same levels of surface MHC class II is that in vivo, as in vitro, *trans-acting* factors (e.g., molecules secreted or released from infected cells that act on uninfected cells) exert potent effects that result in inhibition of IFN-γ-dependent MHC class II expression. We have identified one of these in vitro *trans-acting* factors as IL-6 (38), although we have also observed evidence for inhibition of IFN-γ responses in cultures of macrophages from IL-6 knockout mice, indicating that one or more additional factors exert measurable effects in vitro. An additional possibility is that one or more components of *M. tuberculosis* (in addition to peptidoglycan) inhibit IFN-γ induction of MHC class II in vivo. Because *M. tuberculosis* has been reported to contain one or more agonists of TLR4 (52–54), and because another agonist of TLR4 (LPS) has also been reported to inhibit IFN-γ induction of MHC class II (24, 55, 56), TLR4 signaling may contribute to the TLR2-independent effects observed in our in vivo studies. Our finding of equivalent expression of MHC class II on infected and uninfected recruited macrophages from the lungs of infected mice suggests that *trans-acting* effects also occur in *M. tuberculosis* infection in vivo.
we observed similar expression of MHC class II on infected and uninfected recruited macrophages, it is likely that one or more trans-acting factors contribute to the equivalent expression of MHC class II on TLR2-deficient and TLR2-replete recruited macrophages.

A third potential interpretation of the equivalent expression of MHC class II on TLR2-deficient and TLR2-replete and on directly infected and uninfected recruited macrophages is that M. tuberculosis only inhibits IFN-γ induction of MHC class II in vitro, but not in vivo. There is strong evidence that such mechanisms occur in cultured macrophages, both in primary cells and cell lines. M. tuberculosis and specific bacterial components have been found to inhibit IFN-γ responses in primary human monocyte-derived macrophages (13), phorbol ester-treated THP-1 human monocytic leukemia cells (22), murine RAW264.7 and J774A1 macrophages (19, 25), and murine bone marrow-derived (18, 24, 38) and alveolar macrophages (26). Despite these consistent and robust in vitro findings, the in vivo model may not reflect the complex interplay of cells and soluble factors found in vivo. Nonetheless, we found that recruited interstitial macrophages, which exhibit absolute dependence on IFN-γ responsiveness for MHC class II expression, express 10-fold lower amounts of surface MHC class II than myeloid DC or alveolar macrophages after isolation from the lungs of infected mice (compare Figs. 6, 7, and 8). Because DC and alveolar macrophages are capable of high level expression of MHC class II in the absence of IFN-γ responsiveness (Fig. 3), and because treatment of cultured macrophages with IFN-γ stimulates expression of surface MHC class II to levels equivalent to those on cultured mature DC, the observed 10-fold lower expression of MHC class II on recruited interstitial macrophages compared with lung DC is compatible with inhibition of their responsiveness to IFN-γ. Although our results do not prove that macrophage responses to IFN-γ are impaired during M. tuberculosis infection, they also do not establish that M. tuberculosis cannot inhibit macrophage responses to IFN-γ in vivo.

Our use of GFP-expressing M. tuberculosis also allowed us to examine the role of direct infection in maturation of DC in vivo during M. tuberculosis infection. Our results indicate that at early times after infection (through 21 days), a larger fraction of the DC that contain bacteria has matured compared with the population of uninfected DC, as assessed by surface expression of MHC class II. In contrast, at later time points (28 and 35 days), there was no difference in expression of surface class II on infected and uninfected DC, as assessed by surface expression of MHC class II. Reciprocal expression of surface MHC class II to levels equivalent to those on cultured mature macrophages: comparison of other cytokines with interferon-γ. J. Exp. Med. 160: 600–605.


References


Downloaded from http://www.jimmunol.org/ by guest on April 19, 2017