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Activated Macrophages Infected with *Legionella* Inhibit T Cells by Means of MyD88-Dependent Production of Prostaglandins1

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To understand how macrophages (Mφ) activated with IFN-γ modulate the adaptive immune response to intracellular pathogens, the interaction of IFN-γ-treated bone marrow-derived murine Mφ (BMφ) with *Legionella pneumophila* was investigated. Although *Legionella* was able to evade phagosome lysosome fusion initially, and was capable of de novo protein synthesis within IFN-γ-treated BMφ, intracellular growth of *Legionella* was restricted. It was determined that activated BMφ infected with *Legionella* suppressed IFN-γ production by Ag-specific CD4 and CD8 T cells. A factor sufficient for suppression of T cell responses was present in culture supernatants isolated from activated BMφ following *Legionella* infection. Signaling pathways requiring MyD88 and TLR2 were important for production of a factor produced by IFN-γ-treated BMφ that interfered with effector T cell functions. Cyclooxygenase-2-dependent production of PGs by IFN-γ-treated BMφ infected with *Legionella* was required for inhibition of effector T cell responses. From these data we conclude that activated Mφ can down-modulate Ag-specific T cell responses after they encounter bacterial pathogens through production of PGs, which may be important in preventing unnecessary immune-mediated damage to host tissues. The *Journal of Immunology*, 2005, 175: 8181–8190.

The cell-mediated adaptive immune response is critical in the defense against many bacterial pathogens. Secretion of IFN-γ by Ag-specific CD4 T cells stimulated during the course of infection is important for the activation of macrophages (Mφ).1 IFN-γ activation of Mφ results in the up-regulation of antimicrobial functions, including the production of toxic oxygen and nitrogen radicals, the restriction of nutrients essential to bacterial growth, and production of proinflammatory cytokines (1, 2). Although the activation of Mφ is often critical for the clearance or containment of pathogen growth, the inflammatory response can be damaging to host tissues and must be carefully regulated (3, 4). Thus, the key to a protective immune response to intracellular pathogens involves the activation of effector T cells that can identify host cells that are actively infected, followed by down-regulation of the immune response once activated Mφ have cleared or contained the organism.

*Legionella pneumophila* provides an excellent model organism for investigating how adaptive immune responses to intracellular bacterial pathogens are controlled. *Legionella* is ubiquitous in nature and has the ability to replicate inside most freshwater protozoa (5). *Legionella* has the potential to cause disease in humans when contaminated water sources are aerosolized and inhaled (6, 7). Once inside the lung, the bacterium is internalized by alveolar Mφ. Using strategies that evolved for replication in protozoan hosts, *Legionella* avoids destruction and multiplies within human Mφ (8). A specialized type IVb secretion system called Dot/Icm is essential for *Legionella* intracellular multiplication (9–12). The Dot/Icm system injects bacterial proteins into eukaryotic host cells (13–16). These injected proteins modulate transport of the *Legionella*-containing vacuole, preventing this compartment from fusing with lysosomes and promoting the remodeling of this compartment into an organelle resembling the endoplasmic reticulum (ER) (17–20). It is within this ER-derived organelle that intracellular multiplication of *Legionella* occurs (21).

IFN-γ-activated Mφ are important for clearance of *Legionella* in vivo (22). Ex vivo treatment of murine Mφ and human monocytes with IFN-γ restricts the intracellular growth of *Legionella* (23–25). Studies using electron microscopy have suggested that *Legionella* are able to create an ER-like organelle within IFN-γ-activated Mφ, but the bacteria are unable to replicate within this compartment (23). Although activation of Mφ by IFN-γ is necessary for clearance of infection, prolonged production of IFN-γ by Ag-specific T cells results in damage to host tissues. In this study, we more closely examine interactions between *Legionella* and activated Mφ, and uncover a process by which activated Mφ inhibit secretion of IFN-γ by effector T cells during presentation of *Legionella* Ags.

**Materials and Methods**

**Bacterial cultures**

The *Legionella* serogroup 1 strain, Lp01 (26), and the isogenic *dotA* mutant strain, CR58 (27), were cultured on charcoal yeast extract agar (28) for 2 days before use in experiments. GFP-producing *Legionella* harbored the plasmid pAM239 (17). For experiments examining GFP fluorescence after infection, *Legionella* was grown on plates supplemented with chloramphenicol (6.25 μg/ml), and GFP expression was induced after infection by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mM) to the tissue culture medium.
**Mφ cell cultures**

Unless indicated otherwise, bone marrow-derived murine Mφ (BMφ) were derived from A/J mice (Harlan Sprague-Dawley). The MyD88⁺/⁺ mice (29), TLR2⁻/⁻ mice (30), and TLR4⁻/⁻ (31) mice were described previously and had been backcrossed onto a C57BL/6 background. Cultures of BMφ were prepared as described previously (32).

**Up-regulation of MHC class II by IFN-γ-treated BMφ**

To determine whether BMφ treated with IFN-γ up-regulate MHC class II, BMφ were plated on nontissue culture-treated dishes and treated with 0.0, 0.125, 1.0, or 10.0 ng/ml IFN-γ (1 ng of IFN-γ is equivalent to 10 U of IFN-γ) for 24 h. BMφ were lifted from the dishes. FcRs were blocked with Fc block (BD Pharmingen 553141) and cells were stained with a PE anti-I-Ak Ab (BD Pharmingen 553537), or isotype control (BD Pharmingen 555058) in PBS containing 2% paraformaldehyde and 2% FCS (FACS buffer) for 1 h at 4°C. Cells were washed three times in FACS buffer and analyzed by flow cytometry.

**Single cell assays to measure Legionella internalization and formation of replicative vacuoles**

BMφ were plated at 1.5 × 10⁶ on glass coverslips in 24-well dishes and treated with IFN-γ at 0.125 ng/ml, as indicated. Twenty-four hours later, BMφ were infected with Legionella at a multiplicity of infection (MOI) of 50 for 0.5 h. Wells were then washed three times with PBS to remove extracellular bacteria. Cells were fixed at 1 or 12 h postinfection in PBS containing 2% paraformaldehyde (PFA) for 20 min at room temperature. After fixation, coverslips were washed three times with PBS. Coverslips were permeabilized and blocked in PBS containing 2% goat serum and 0.2% saponin (perm-block solution) for 15 min at room temperature. Coverslips were then incubated overnight at 4°C in perm-block containing mouse anti-Legionella polyclonal Ab. Coverslips were washed three times in PBS. Coverslips were incubated 1 h at 37°C with a goat anti-mouse secondary Ab (Alexa Fluor 546) in perm-block solution, and then washed three times in PBS. Coverslips were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize host and bacterial DNA, and washed three times in PBS. Coverslips were mounted on slides and examined by fluorescence microscopy. A replicative phagosome is defined as a vacuole containing four or more Legionella after 12 h postinfection. Most replicative phagosomes examined contained >10 bacteria, indicating several rounds of replication had occurred. The efficiency of replicative phagosome formation was determined by dividing the percentage of host cells containing replicative vacuoles at 12 h by the percentage of cells containing intracellular Legionella 1 h after infection. At least 300 BMφ were scored at each time point for each independent assay. The assay was repeated three times, and the data represent the average percentage of replicative phagosome formation ± SD.

**Intracellular growth curves**

Growth of Legionella in BMφ was measured, as described previously (27). To assess the effect of IFN-γ on the intracellular growth of Legionella, BMφ were treated with IFN-γ overnight at 0.0, 0.125, 1.0, and 5.0 ng/ml, and infected the following day. Data are normalized to number of bacteria internalized and are presented as mean CFU per well for the indicated times from three independent wells ± SD.

**Phagosome transport and intracellular synthesis of GFP by Legionella**

Lyosomal-associated membrane protein 1 (LAMP-1) acquisition by phagosomes containing Legionella and de novo synthesis of GFP by intracellular Legionella were examined, as described previously (33, 34). Briefly, BMφ were plated on glass coverslips and treated with IFN-γ, as described above. The following day, BMφ were infected with the wild-type or dotA mutant strains of Legionella harboring pAM239 at an MOI of 100 for 0.5 h. Cells were then fixed in PFA, as described above. BMφ were stained for LAMP-1 using Ab 1D4B (35), and for DNA with 0.1 µg/ml DAPI. Cells were examined by fluorescence microscopy to determine whether each bacterial localization with LAMP-1 and de novo synthesis of GFP by intracellular Legionella.

**Assays to measure presentation of Legionella Ags**

Legionella-specific CD4 T cells were produced, as described previously (34). Legionella-specific CD8 T cells were produced similarly, using Miltenyi Biotec MACS CD8 magnetic beads (494-01) for positive selection.

For Ag presentation assays, BMφ were replated at a density of 1 × 10⁶ in 96-well flat-bottom tissue culture dishes. Where indicated, BMφ were treated overnight with IFN-γ (BD Pharmingen 554587) at the indicated concentration. Cells were infected with Legionella at an MOI of 20. After addition of bacteria, plates were centrifuged at 150 × g for 5 min to enhance contact of the bacteria with the BMφ. Plates were then floated in a 37°C water bath for 15 min before extracellular bacteria and IFN-γ were removed by washing wells three times with PBS. T cells were added in T cell medium (RPMI 1640 containing 10% FBS, 1% MEM nonessential amino acids, 1% MEM essential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 µM 2-ME, 10 mM HEPES (pH 7.55) at a concentration of 5 × 10⁵ per well or 3 × 10⁴ per well for CDB T cells. After 48 h of incubation, T cell responses were measured by determining the amount of IFN-γ present in culture medium using an ELISA. Data represent the average of IFN-γ concentration for at least three independent wells ± SD.

For assays in which BMφ were fixed with PFA, BMφ were treated and infected as described above. At 10 h after infection, wells were fixed with 2% PFA for 20 min at room temperature. Wells were then washed four times with PBS. To quench any reactive PFA remaining, wells were incubated with medium containing 20% FBS overnight. T cells were added the following day at a concentration of 5 × 10⁵ per well.

**Blocking programmed death 1 (PD-1) ligand 2 (PD-L2) interaction with PD-1**

To block PD-L2 interactions with PD-1, BMφ were infected, as described above. After washing wells to remove uninternalized bacteria, a blocking anti-PD-L2 Ab (eBiosciences 16-5986), or isotype control (eBiosciences 16-4321) was added to the wells at a concentration of 10 µg/ml. After 0.5 h of incubation, T cells were added to wells containing infected BMφ and blocking Ab.

**Conditioned medium**

To make conditioned medium, BMφ were treated with IFN-γ at the indicated concentrations. Twenty-four hours later, BMφ were infected with the dotA mutant strain of Legionella at MOI 20, centrifuged for 5 min at 150 × g, and floated in a 37°C water bath for 15 min. Wells were then washed three times with PBS to remove uninternalized bacteria and IFN-γ. Plates were then incubated for 24 h. Supernatants were then removed and frozen at −20°C for later use. Where indicated, conditioned medium was treated with proteinase K (100 µg/ml) for 30 min at 37°C and then boiled for 10 min to inactivate the protease. Conditioned medium was made in the presence of cyclooxygenase (COX) inhibitors by incubating IFN-γ-treated BMφ with either 0.015% DMSO (solvent control), 1 µM indomethacin (COX1/COX2 inhibitor; Cayman Chemical 70270), or 5 µM NS-398 (COX2 inhibitor; Cayman Chemical 70590) for 5 h before infection with dotA mutant Legionella (MOI 20).

**ELISAs and enzyme immunoassay**

IFN-γ was quantified using BD Pharmingen 551216 (capture) and BD Pharmingen 554410 (detection), as per manufacturer’s instructions. PGE2 was measured using a PGE2 enzyme immunoassay kit (Cayman Chemical 514010), as per the manufacturer’s instructions. Data represent the average IFN-γ or PGE₂ concentration for at least three independent wells ± SD.

**Inhibition of NO production by activated BMφ**

To inhibit NO production, N⁴-methyl-L-arginine (LMNA; Sigma-Aldrich) was added to cultures of infected BMφ and CD4 T cells that were either untreated, treated with IFN-γ, or treated with conditioned medium, at a concentration of 1 mM.

**Statistical analysis**

A Student’s two-tailed t test with unequal variance was used to determine the statistical significance of the results. Value of p < 0.05 is indicated by ∗; p < 0.005 is indicated by ∗∗.

**Results**

Legionella traffics correctly, but does not replicate in IFN-γ-treated BMφs

Murine Mφ have been used extensively to study intracellular transport and growth of Legionella (36). To investigate the role of activated Mφ in containment and clearance of intracellular pathogens, we began investigating interactions between IFN-γ-activated BMφ and Legionella. The responsiveness of BMφ to IFN-γ was
assessed after overnight treatment with the indicated concentrations of IFN-γ. After 24 h, BMφ were stained with an Ab against MHC class II molecules and analyzed by flow cytometry (Fig. 1A). IFN-γ treatment resulted in a dose-dependent increase in MHC class II staining, indicating that BMφ were responding to IFN-γ added exogenously. Next, replication of Legionella in IFN-γ-treated BMφ was assessed both by measuring the formation of vacuoles that contain replicating Legionella using microscopy and by measuring bacterial numbers directly after plating Legionella on agar medium and counting CFU. These data show that treating BMφ with as little as 0.125 ng/ml IFN-γ renders these cells non-permissive for Legionella in intracellular multiplication. Fewer than 10% of all internalized Legionella bacteria were found in vacuoles that supported intracellular growth in IFN-γ-treated BMφ, whereas greater than 70% of the internalized Legionella formed replicative organelles in the untreated BMφ (Fig. 1B). CFU-based studies confirmed that Legionella growth was restricted in IFN-γ-treated BMφ (Fig. 1C).

Fluorescence microscopy was used to investigate whether IFN-γ affects the transport of vacuoles containing Legionella in BMφ. Most vacuoles containing wild-type Legionella did not stain positive for the late-endosomal/lysosomal protein LAMP-1 in either IFN-γ-treated or untreated BMφ, indicating that Legionella retains the ability to evade fusion with lysosomes in IFN-γ-treated BMφ (Fig. 1D, upper panels). Legionella was seen replicating in the LAMP-1-negative vacuoles present in the untreated BMφ, whereas the LAMP-1-negative vacuoles found in the IFN-γ-treated BMφ were unable to support Legionella replication. As expected, Legionella dotA mutant bacteria were found in LAMP-1-positive vacuoles in both IFN-γ-treated and untreated BMφ (Fig. 1D, lower panels).

Because previous studies using dendritic cells (DCs) indicated that bacterial protein synthesis after internalization was essential for optimal stimulation of CD4 T cells (34), de novo protein synthesis of bacterial proteins within activated BMφ was investigated. Bacteria used in these studies contained a plasmid encoding GFP under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. Legionella containing this plasmid are not detectable by fluorescence microscopy without induction of GFP on IPTG-containing medium (34). IPTG was added to tissue culture medium after infection to test whether internalized bacteria that were initially grown under noninducing conditions were residing in a compartment that allowed de novo synthesis of Legionella proteins, including GFP. Wild-type Legionella in both

**FIGURE 1.** IFN-γ-treated BMφ restrict Legionella growth. A, Surface staining of MHC class II on BMφ was measured by FACS. Shown are data from untreated BMφ stained with a PE-labeled isotype control Ab (black), untreated BMφ stained with PE-labeled anti-I-A^k Ab (red), and BMφ stained with PE-labeled anti-I-A^k after treatment for 24 h with the concentrations of IFN-γ indicated (0.125 ng/ml, green; 1.0 ng/ml, blue; 10.0 ng/ml, violet). The efficiency of replicative vacuole (RV) formation was determined for Legionella after internalization by BMφ. The RV index represents the percentage of Legionella internalized by the BMφ that went on to establish a vacuole permissive for replication. Data show that most Legionella were successful in establishing an RV in untreated BMφ (0.0 ng/ml), but were unable to establish an RV in BMφ treated with 0.125 ng/ml IFN-γ. C, Growth of Legionella in BMφ was measured by determining the increase in CFUs at the times indicated. Shown are the growth kinetics for Legionella in untreated BMφ (red) and BMφ treated for 24 h with 0.125 ng/ml IFN-γ (green), 1.0 ng/ml IFN-γ (blue), and 5.0 ng/ml IFN-γ (violet). D, Untreated BMφ (No IFN-γ) and BMφ treated with 0.125 ng/ml IFN-γ (+ IFN-γ) were infected with wild-type (top panels) or dotA mutant (bottom panels) strains of Legionella harboring pAM239, a plasmid with an IPTG-inducible gene encoding GFP. After infection, IPTG was added to the medium for 5 h, and cells were fixed and then stained with a LAMP-1-specific Ab (red) to identify late endosomes and lysosomes: DAPI (blue) to identify host and bacterial DNA, and GFP fluorescence (green) to detect de novo protein synthesis intracellularly. For each panel, there is a large merged color image having a boxed region of interest. In the lower portion of each panel, the boxed region of interest has been magnified to show the individual gray scale images of DNA, GFP, and LAMP-1 staining of the region and the merged color image of the region.
IFN-γ-treated and untreated BMφ produced GFP following infection, indicating that these bacteria reside in vacuoles that allow synthesis of new proteins (Fig. 1D, upper panels). The Legionella dotA mutant was unable to synthesize GFP in either IFN-γ-treated or untreated BMφ, consistent with residence in an endocytic vacuole (Fig. 1D, lower panels). These studies indicate that IFN-γ-treated BMφ restrict the growth of Legionella by a process that does not involve altering vacuole transport or de novo synthesis of bacterial proteins intracellularly.

**IFN-γ-treated BMφ inhibit effector T cell responses following Legionella infection**

Treatment of BMφ with IFN-γ increased surface presentation of MHC class II molecules, which might enhance CD4-mediated T cell responses to Legionella Ags. T cell responses to IFN-γ-treated and untreated BMφ infected with Legionella were compared. Ag-specific responses were assayed following infection of BMφ with Legionella by measuring levels of IFN-γ that traffics to an ER-derived compartment, and when IFN-γ-treated Legionella were infected with a dotA mutant strain that resides in a LAMP-1-positive vacuole. Thus, IFN-γ-treated BMφ that had been purified from mice that had been immunized with wild-type Legionella. Contrary to what was predicted, Legionella-specific T cell responses were diminished when BMφ treated with IFN-γ were used as presenting cells (Fig. 2A). Ag-specific T cell responses were reduced both when the IFN-γ-treated BMφ were infected with a wild-type strain of Legionella that traffics to an ER-derived compartment, and when IFN-γ-treated Legionella were infected with a dotA mutant strain that resides in a LAMP-1-positive vacuole. Thus, IFN-γ-treated BMφ inhibited T cell responses regardless of the intracellular transport pathway used by Legionella.

It was unlikely that reduced T cell responses resulted from Legionella growth restriction by IFN-γ-treated BMφ because Legionella dotA mutants that do not replicate intracellularly were also less efficient at stimulating Legionella-specific T cells following uptake by IFN-γ-treated BMφ. However, to more carefully address whether the diminished T cell response to IFN-γ-treated BMφ might be due to restriction of Legionella intracellular multiplication, IFN-γ-treated and untreated BMφ were infected with Legionella strains derived from a thyA mutant, which are thymidine auxotrophs. BMφ were infected in the presence or absence of thymidine in a tissue culture medium. Legionella thyA mutants create ER-derived organelles in BMφ, but replication is impaired unless thymidine is added to the tissue culture medium (26). The T cell response was significantly higher when untreated BMφ infected with a replication-deficient thyA mutant strain were used as presenters compared with the response detected when IFN-γ-treated BMφ infected with the same strain were used as presenters. Moreover, T cell responses were similar regardless of whether thymidine was added to the medium (Fig. 2B). These data indicate that the diminished T cell response observed when using IFN-γ-treated BMφ as presenting cells was not due to restriction of Legionella intracellular multiplication.

Ag presentation assays were performed using CD8 T cells from immunized mice to determine whether responses to Legionella Ags presented on MHC class I molecules are diminished when IFN-γ-treated Mφ infected with Legionella are used as presenting cells. Similar to what was observed using CD4 T cells, Legionella-specific CD8 T cell responses were diminished when using IFN-γ-treated BMφ as presenters (Fig. 2C). In addition, these data indicate that CD8 T cells respond better to BMφ infected with wild-type Legionella compared with dotA mutant bacteria, which is most likely due to an increase in the abundance of substrates that are available for MHC class I presentation resulting from the injection of bacterial proteins into the cytosol by wild-type Legionella. From these data, it can be concluded that IFN-γ-treated BMφ infected with Legionella suppress Ag-specific effector responses by both CD4 and CD8 T cells.

**A factor that inhibits effector T cell responses is released by IFN-γ-treated BMφ after Legionella infection**

The surface protein PD-L2 is up-regulated and expressed on Mφ after stimulation with IFN-γ (37). Importantly, PD-L2 can interact...
with PD-1 on activated T cells, and this interaction inhibits T cell effector functions, including the secretion of IFN-γ (38). A PD-L2-specific blocking Ab was used to investigate whether the expression of PD-L2 on the surface of IFN-γ-treated BMφ was interfering with Legionella-specific T cell responses. When PD-L2 blocking Ab was added to untreated BMφ infected with Legionella, the CD4 T cell response was enhanced nearly 2-fold (Fig. 3A). Blocking PD-L2 on IFN-γ-treated BMφ also resulted in a 2-fold increase in the Legionella-specific CD4 T cell response; however, blocking the PD-L2/PD-1 interaction did not restore the T cell response to the level observed when untreated BMφ were used as presenting cells. Thus, it is unlikely that negative costimulation resulting from PD-L2/PD-1 interactions is the primary mechanism by which IFN-γ-treated BMφ inhibit Legionella-specific T cell responses.

To test whether additional proteins on the surface of IFN-γ-activated BMφ could be involved in suppressing T cell function, Legionella-infected BMφ were fixed gently using paraformaldehyde (PFA) and then used as presenting cells in conjunction with responder CD4 T cells from immunized mice. These data show that when IFN-γ-treated BMφ and untreated BMφ infected with Legionella were fixed before the addition of T cells, equivalent Ag-specific T cell responses were measured (Fig. 3B). Thus, it is unlikely that the primary determinant responsible for interfering with effector T cell functions is a cell surface protein on BMφ up-regulated upon treatment of cells with IFN-γ.

To determine whether IFN-γ-treated BMφ release a soluble factor that inhibits T cell responses, conditioned supernatants were collected from infected and uninfected BMφ and assayed for an activity that interfered with Legionella-specific T cell responses. Indeed, conditioned supernatants collected from IFN-γ-treated BMφ infected with Legionella dotA mutant bacteria had an activity that inhibited a Legionella-specific CD4 T cell response (Fig. 3C). In contrast, supernatants isolated from uninfected IFN-γ-treated BMφ did not interfere with a Legionella-specific CD4 T cell response. Conditioned supernatants from IFN-γ-treated BMφ treated with wild-type L. pneumophila were able to inhibit effector T cell responses similarly to the supernatants collected from activated BMφ infected with the dotA mutant (data not shown). These data indicate that Legionella infection of IFN-γ-treated BMφ results in the release of a factor(s) that can inhibit effector T cell functions, and consistent with the data in Fig. 2A, production of this factor is independent of bacterial proteins translocated into host cells by the Dot/Icm system.

BMφ require both IFN-γ activation and stimulation of a MyD88-dependent pathway to generate a response that inhibits effector T cell functions

Given that IFN-γ-treated BMφ release a factor that inhibits effector T cell functions only after they are infected with Legionella, the possibility that production of this factor requires activation of TLRs was investigated. If BMφ require TLR signaling to produce a factor that inhibits T cell function, then the adapter protein MyD88 should be important for this response (39, 40). Thus, supernatants from IFN-γ-treated BMφ derived from C57BL/6 mice deficient for MyD88 was assayed for a factor that inhibits effector T cell functions. Increased surface presentation of MHC class II molecules indicated that MyD88-deficient BMφ were responding to the addition of IFN-γ (data not shown); however, culture supernatants from IFN-γ-treated MyD88-deficient BMφ infected with Legionella was unable to inhibit effector T cell functions (Fig. 4A). Thus, MyD88 is required for production of a factor that inhibits effector T cells upon Legionella infection of IFN-γ-treated BMφ.

**FIGURE 3.** Legionella infection of IFN-γ-treated BMφ triggers secretion of a factor that inhibits T cell responses. Ag presentation assays were conducted to measure CD4 T cell responses to BMφ infected with Legionella. A, BMφ pretreated with the concentrations of IFN-γ indicated on the x-axis and infected with wild-type Legionella were used as presenting cells. CD4 T cell responses are indicated on the y-axis as the amount of IFN-γ secreted after coinoculation with infected BMφ. Before the addition of T cells, a control Ab (■) or a PD-L2 blocking Ab (□) was added to the presentation assay. Significant differences between control and anti-PD-L2-treated BMφ are indicated. B, BMφ pretreated with the concentrations of IFN-γ indicated on the x-axis and infected with wild-type Legionella were used as presenting cells. CD4 T cell responses are indicated on the y-axis as the amount of IFN-γ secreted after coinoculation with infected BMφ. No significant differences in T cell responses were detected. C, Variables used in preparation of the conditioned supernatants are indicated on the x-axis and include the amount of IFN-γ added during the pretreatment of BMφ and infection of BMφ with the Legionella dotA mutant strain. These conditioned supernatants were added to a standard Ag presentation assay in which CD4 T cell responses to BMφ infected with wild-type Legionella were measured. CD4 T cell responses are indicated on the y-axis as the amount of IFN-γ secreted after coinoculation with infected BMφ. Significant differences in CD4 T cell responses compared with control presentation assays containing supernatant from untreated BMφ (IFN-γ 0.0, Legionella −) are indicated.

Legionella is a weak activator of TLR4, but a potent activator of TLR2 (41). BMφ from C57BL/6 mice deficient in either TLR2 or TLR4 were used to investigate whether TLR signaling plays an
important role in the inhibition of T cell responses by IFN-γ-treated BMφ infected with *Legionella*. Consistent with *Legionella* being a poor activator of TLR4, BMφ deficient in TLR4 did not show a significant difference in production of the T cell-suppressive factor after *Legionella* infection (Fig. 4B). In contrast, the T cell inhibitory activity was significantly reduced in conditioned medium harvested from TLR2-deficient BMφ, consistent with TLR2 being important for MyD88-dependent pathways of innate immune detection of *Legionella*. Although reduced, the inhibitory activity in conditioned medium from TLR2-deficient BMφ was still greater than in conditioned medium from MyD88-deficient BMφ, suggesting that *Legionella* stimulation of other TLR molecules can also activate this MyD88-dependent response.

A protease-resistant factor released by IFN-γ-treated BMφ is sufficient to suppress T cell functions

Previous reports suggest that NO produced by activated BMφ can mediate suppression of T cell responses (42) as well as induce apoptosis of T cells (43). To determine whether NO-mediated events were contributing to the diminished T cell responses, NO production was inhibited by the addition of L-MNA to cultures of infected BMφ and T cells. Addition of L-MNA to BMφ infected with *Legionella* did not affect the T cell response when compared with controls in which L-MNA was absent, and conditioned me-

PGs produced upon *Legionella* infection of activated BMφ inhibit effector T cell function

PGs, which are derived from membrane phospholipids, have been demonstrated to affect the function of many immune system cells (44). PGE₂, in particular, is released by Mφ in response to LPS, IL-1, and TNF-α, and has been demonstrated to down-modulate the function of Th1 T cells, including the secretion of IFN-γ by these cells (44, 45). To determine whether PGE₂ production could account for the suppression of T cell functions, PGE₂ levels were measured in culture supernatants of BMφ that were either treated with IFN-γ, infected with *Legionella*, or both (Table I). Untreated BMφ, BMφ treated with only IFN-γ, and BMφ infected with *Legionella* produced very low levels of PGE₂ (<100 pg/ml). In contrast, BMφ that were treated with IFN-γ and infected with *Legionella* produced high amounts of PGE₂. Given the observation that conditioned supernatants isolated from IFN-γ-treated BMφ from MyD88-deficient mice did not inhibit T cell responses, supernatants from MyD88-deficient BMφ were also assayed for PGE₂ levels. These data show that at all IFN-γ concentrations tested, PGE₂ levels did not increase following *Legionella* infection of MyD88-deficient BMφ (Fig. 6A). Thus, the pattern of PGE₂ production observed in these experiments is consistent with PGE₂ in BMφ supernatants being involved in inhibition of T cell function.

To test whether PGs produced by activated BMφ were important for suppression of effector T cell functions, COX inhibitors were used to block PG production. Supernatants were collected from IFN-γ-treated BMφ infected with *Legionella* that had been incubated in the presence of either indomethacin, an inhibitor of both COX-1 and COX-2, or NS-398, a COX-2 inhibitor. As shown in Table I, these COX inhibitors were effective in blocking PGE₂ production by IFN-γ-treated BMφ infected with *Legionella*. Conditioned medium from BMφ treated with COX inhibitors were tested to determine whether these supernatants retained an activity that could suppress T cell responses. Medium collected from IFN-γ-treated BMφ infected with *Legionella* receiving the solvent DMSO was effective at inhibiting T cell responses when compared with medium from BMφ that did not receive IFN-γ and *Legionella* (Fig. 6B). In contrast, medium harvested from IFN-γ-treated BMφ infected with *Legionella* receiving either indomethacin or NS-398 failed to suppress T cell secretion of IFN-γ (Fig. 6B). Thus, PGs released into the culture medium by activated BMφ were required for the activity in these supernatants that suppressed secretion of IFN-γ by effector T cells.

Discussion

Mφ recruited to sites of microbial infection present Ags to effector T cells. Upon recognition of a cognate MHC-Ag complex, effector T cells will activate Mφ through the production of IFN-γ (46). Although Mφ activation is essential for the elimination of most pathogens, sustained production of IFN-γ can have detrimental
At the cell biological level, we found that *Legionella* was initially successful at evading phagosome-lysosome fusion within BMφ. Our observation that *Legionella* replication is restricted in IFN-γ-treated BMφ is consistent with previous reports (23–25, 51). Previous studies using BMφ from A/J mice indicate that activated BMφ restrict the growth of *Legionella* by a process that does not require production of reactive oxygen and nitrogen species and that is in part mediated by the sequestration of iron (51). Although the infection of IFN-γ-treated BMφ by *Legionella* mirrors the infection of murine DCs by *Legionella* at the cell biological level (34), at the immunological level important differences were observed in the abilities of IFN-γ-treated BMφ to activate *Legionella*-specific T cell responses compared with what has been observed for DCs. *Legionella*-specific CD4 and CD8 T cells responded poorly in Ag presentation assays when infected IFN-γ-treated BMφ were used as presenting cells, whereas live DCs infected with *Legionella* were previously shown to be potent effectors on the host, including tissue damage (47–50). It is predicted that BMφ should have the ability to down-regulate IFN-γ production by effector T cells after they have reached a stage of activation that allows them to eliminate a pathogen at the site of infection. Indeed, a number of in vivo studies have indicated important roles for myeloid cells and IFN-γ in T cell peripheral tolerance to microbial infection (42–45). Our investigations of *Legionella* interactions with IFN-γ-treated BMφ have provided an ex vivo system to investigate the mechanisms by which BMφ can induce peripheral tolerance and how these activities are controlled.

At the cell biological level, we found that *Legionella* was initially successful at evading phagosome-lysosome fusion within IFN-γ-treated BMφ and produced new proteins, but the bacteria failed to replicate within the vacuole they established in activated BMφ. Our observation that *Legionella* replication is restricted in IFN-γ-treated BMφ is consistent with previous reports (23–25, 51). Previous studies using BMφ from A/J mice indicate that activated BMφ restrict the growth of *Legionella* by a process that does not require production of reactive oxygen and nitrogen species and that is in part mediated by the sequestration of iron (51). Although the infection of IFN-γ-treated BMφ by *Legionella* mirrors the infection of murine DCs by *Legionella* at the cell biological level (34), at the immunological level important differences were observed in the abilities of IFN-γ-treated BMφ to activate *Legionella*-specific T cell responses compared with what has been observed for DCs. *Legionella*-specific CD4 and CD8 T cells responded poorly in Ag presentation assays when infected IFN-γ-treated BMφ were used as presenting cells, whereas live DCs infected with *Legionella* were previously shown to be potent effectors on the host, including tissue damage (47–50). It is predicted that BMφ should have the ability to down-regulate IFN-γ production by effector T cells after they have reached a stage of activation that allows them to eliminate a pathogen at the site of infection. Indeed, a number of in vivo studies have indicated important roles for myeloid cells and IFN-γ in T cell peripheral tolerance to microbial infection (42–45). Our investigations of *Legionella* interactions with IFN-γ-treated BMφ have provided an ex vivo system to investigate the mechanisms by which BMφ can induce peripheral tolerance and how these activities are controlled.

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Table I. *PGE2* levels (pg/ml) produced by BMφs in response to treatment with IFN-γ and *Legionella* infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitor</th>
</tr>
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<tbody>
<tr>
<td>ng/ml IFN; MOI</td>
<td>DMSO</td>
</tr>
<tr>
<td>0.0; 0</td>
<td>Below limit&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.125; 0</td>
<td>156 ± 47 pg/ml</td>
</tr>
<tr>
<td>0.0; 20</td>
<td>158 ± 18 pg/ml</td>
</tr>
<tr>
<td>0.125; 20</td>
<td>4932 ± 990 pg/ml</td>
</tr>
</tbody>
</table>

<sup>a</sup>Solvent control.
<sup>c</sup>Limit of detection for assay was 85 pg/ml.
activators of Ag-specific CD4 T cells (34). In contrast to live cells, fixed IFN-γ-treated BMφ were efficient at stimulating Legionella-specific T cell responses, which suggested that live BMφ produce a secreted factor that inhibits T cell functions. This hypothesis was confirmed using culture supernatants isolated from IFN-γ-treated BMφ infected with Legionella. Production of the inhibitory factor required MyD88, which indicates that both TLR signaling and costimulatory molecules that interact with CD28 and CTLA-4. Although the interaction of B7 molecules with CD28 is stimulatory, the interaction of CTLA-4 with B7 molecules is inhibitory. More recent studies have revealed the existence of other molecules involved in negative costimulation (52). Activated T cells express PD-1, a molecule with homology to CD28 (53). This molecule interacts with PD-L1 and PD-L2, ligands on professional APCs that were identified by their homology to B7 molecules (54, 55). The interaction of PD-L1 and PD-L2 with PD-1 results in the inhibition of T cell responses. From these data, we conclude that live Mφ are able to control T cell responses by a process that is tightly controlled both by the presence of the pathogen and the concentration of IFN-γ in the surrounding environment.

Mφ play a number of important roles in shaping and regulating the immune response. Many of the proteins expressed by Mφ upon stimulation with IFN-γ are involved in modulating adaptive immune responses. Activated Mφ express B7.1 and B7.2, important costimulatory molecules that interact with CD28 and CTLA-4. Although the interaction of B7 molecules with CD28 is stimulatory, the interaction of CTLA-4 with B7 molecules is inhibitory. More recent studies have revealed the existence of other molecules involved in negative costimulation (52). Activated T cells express PD-1, a molecule with homology to CD28 (53). This molecule interacts with PD-L1 and PD-L2, ligands on professional APCs that were identified by their homology to B7 molecules (54, 55). The interaction of PD-L1 and PD-L2 with PD-1 results in the inhibition of T cell responses. Our data indicate that blockage of PD-L2 interaction with PD-1 had a modest effect on enhancing effector T cell responses to Legionella-infected BMφ, although blocking PD-L2 did not restore T cell responses to IFN-γ-treated BMφ to levels detected for untreated BMφ. These data indicate that in addition to its role in tolerance and autoimmunity, PD-L2 is involved in modulating immune responses to bacterial pathogens.
treated with COX inhibitors no longer suppressed T cell secretion of IFN-γ. Additionally, we found that PGE2 levels did not increase in conditioned supernatants from IFN-γ-treated BMφ derived from MyD88-deficient mice following Legionella infection, and these supernatants were unable to suppress T cell responses. The fact that NS-398, which specifically inhibits COX-2, blocks production of the suppressive factor demonstrates that induced expression of the COX-2 enzyme is required for inhibition of T cell function. In conclusion, these data show that activated BMφ can interfere with IFN-γ secretion by effector T cells by a process that involves regulated production of PGE2.

An effective immune response that results in the containment and/or clearance of pathogens that replicate in nondegradative compartments often requires the activation of BMφ by IFN-γ secreted by activated T cells. So, how would down-regulation of T cell function by activated BMφ be beneficial to the host? The immune response to many intracellular pathogens is itself a threat to the host. The damage that results from the inflammatory response and the activation of BMφ can result in fibrosis and scarring of tissue at the site of infection. For example, the scarring of the fallopian tubes in response to Chlamydia infections is mediated primarily by the immune response (3). Likewise, the immune response that contains an infection by Mycobacterium tuberculosis also results in damage, necrosis, and scarring of lung tissue (4). Control of the immune response is crucial to prevent excessive tissue damage, and there are multiple mechanisms in place to ensure that the inflammatory response is down-regulated as infection is cleared. Our data indicate that secretion of PGE2 by activated BMφ and the subsequent inhibition of IFN-γ secretion by T cells may be an important mechanism designed to limit pathogen-specific Th1 responses before they become pathological to the host.

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Disclosures

The authors have no financial conflict of interest.

References