Immunity, Vol. 18, 813–823, June, 2003, Copyright ©2003 by Cell Press

Legionella Reveal Dendritic Cell Functions that Facilitate Selection of Antigens for MHC Class II Presentation

Annie L. Neild and Craig R. Roy*
Section of Microbial Pathogenesis
Boyer Center for Molecular Medicine
Yale University School of Medicine
295 Congress Avenue
New Haven, Connecticut 06536

Summary

To understand how adaptive immune responses are generated against bacteria that avoid being delivered to lysosomes, interactions between professional antigen-presenting cells (APCs) and the intracellular pathogen *Legionella pneumophila* were examined. In contrast to murine bone marrow-derived macrophages (BMMs), we show that dendritic cells (DCs) restrict the growth of intracellular *Legionella*. Similar to what has been reported in BMMs, phagosomes containing *Legionella* matured into endoplasmic reticulum (ER)-derived organelles after DC internalization. Biogenesis of an ER-derived vacuole did not effectively sequester *Legionella* antigens from presentation on MHC class II molecules (MHC II). It was determined that proteins synthesized after *Legionella* had established residence in an ER-derived vacuole were presented by infected APCs. These data indicate that the ability of DCs to restrict intracellular growth of *Legionella* could be an important property that facilitates priming of protective T cell-mediated immune responses to vacuolar pathogens.

Introduction

A common strategy employed by bacterial pathogens to infect eukaryotic host cells is to establish residence in a membrane-bound vacuole that does not fuse with lysosomes (Sinai and Joiner, 1997). This strategy has been adopted by *Chlamydia pneumoniae*, *Legionella pneumophila*, and *Mycobacterium tuberculosis*, which are all examples of bacterial respiratory pathogens that can multiply within nondegradative organelles inside alveolar macrophages. In theory, residence in a nondegradative vacuole should restrict the processing and presentation of bacterial-derived peptides on MHC II, limiting the activation of CD4 T cells having receptors that recognize the antigen-MHC II complex. However, for each of these pathogens, a robust CD4-mediated T cell response is generated during infection (Friedman et al., 1988; Halme and Surcel, 1997; Orme et al., 1993).

To understand immunity to these pathogens, it is important to determine how T cell responses are primed and the mechanism by which bacterial antigens become available for presentation on MHC II. These questions have been addressed primarily by examining the interactions between vacuolar pathogens and macrophages. However, DCs are likely to play an important role in the initiation of the adaptive immune response to these bacteria. DCs have a number of traits that make them well-suited to the task of priming an adaptive immune response against bacterial pathogens. Immature DCs are phagocytic, enabling them to internalize bacteria that have invaded peripheral tissues and mucosal surfaces (Albert et al., 1998; Inaba et al., 1993; Reis e Sousa et al., 1993). Bacterial-derived components, such as lipopolysaccharide (LPS), trigger DC maturation (Mellman et al., 1998). As DCs mature, their phagocytic capacity is reduced and they migrate from peripheral sites to secondary lymphoid organs. Mature DCs secrete IL-12 and other cytokines that play an important role in skewing development of naive T cells toward a TH-1 phenotype (Heufler et al., 1996; Macatonia et al., 1995; Seder et al., 1993). In addition, surface levels of MHC II and costimulatory molecules increase during DC maturation (Mellman et al., 1998; Pierre et al., 1997). These properties make DCs one of the few cell types that can stimulate development of naive CD4 T cells into TH-1 type effectors (Inaba and Steinman, 1985; Steinman, 1991). In the context of microbial infection these events lead to the initiation of a cellular immune response that can respond to bacterial-derived peptides presented on MHC II.

In this study, *Legionella* was used as a model organism to dissect the mechanism by which adaptive immune responses are generated against vacuolar pathogens. *Legionella* is the etiological agent of a severe bacterial pneumonia known as Legionnaires' disease (Fraser et al., 1977). Upon entry into host cells, *Legionella* modulates phagosome transport to prevent the formation of a degradative phagolysosome (Horwitz, 1983b). As they are transported, phagosomes containing *Legionella* associate with vesicles exiting the ER (Kagan and Roy, 2002) and are converted into a ribosome-lined organelle that supports intracellular replication (Horwitz, 1983a; Tilney et al., 2001). The ability of *Legionella* to evade transport to lysosomes and create an ER-derived vacuole requires a bacterial protein secretion apparatus encoded by the *dot* and *icm* genes (Segal et al., 1998; Vogel et al., 1998). The Dot/Icm secretion system injects bacterial proteins into eukaryotic host cells during infection that direct transport of *Legionella*-containing phagosomes to the ER (Nagai et al., 2002; Tilney et al., 2001). *Legionella* mutants defective in this *dot/icm*-encoded secretion system do not replicate intracellularly because they are unable to modulate phagosome transport and reside in conventional phagosomes that are rapidly transported to lysosomes (Berger and Isberg, 1993; Roy et al., 1998). Using wild-type *Legionella* and a *dotA* mutant strain, isogenic bacteria that reside in different subcellular organelles, we set out to determine the extent to which residence in an ER-derived vacuole shelters *Legionella* antigens from processing and presentation on MHC II, events critical for the initiation of adaptive immunity.

*Correspondence: craig.roy@yale.edu

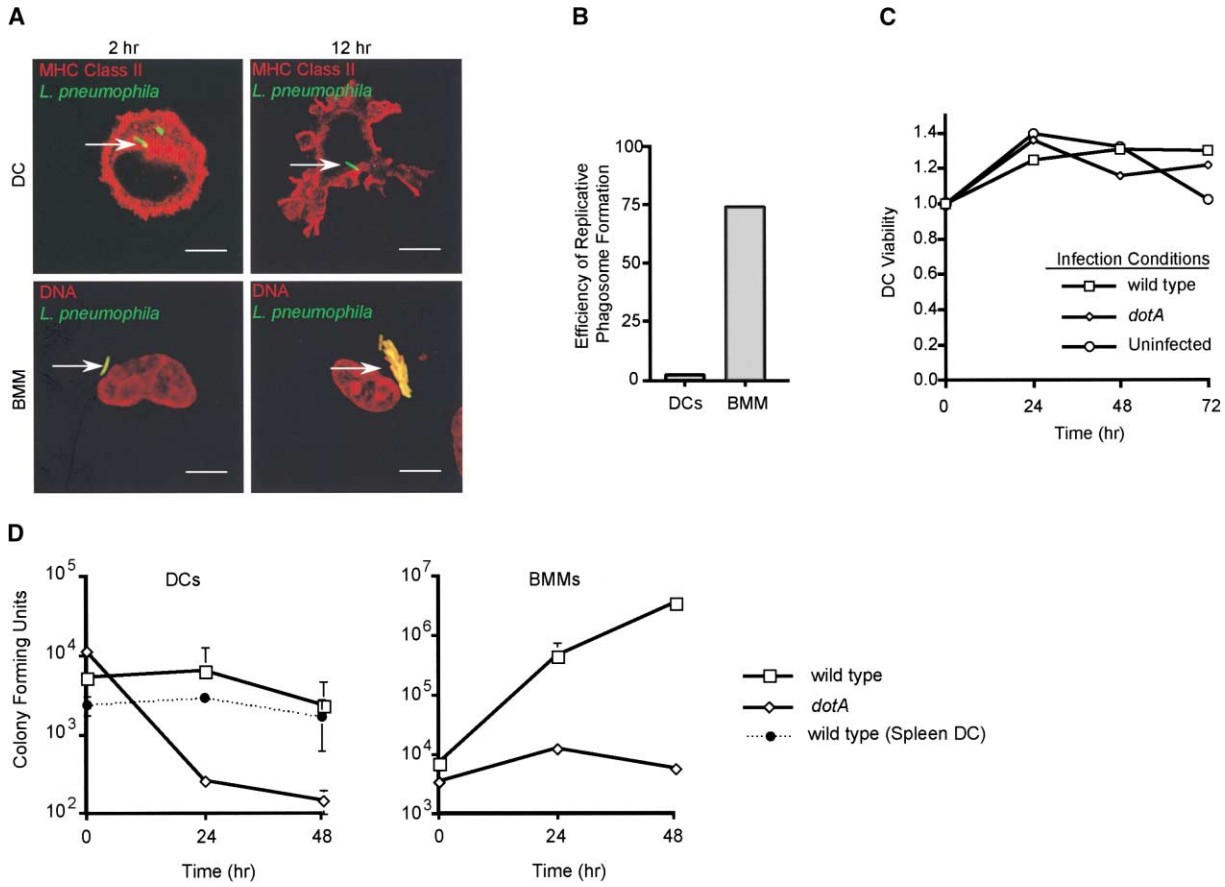

Figure 1. DCs Restrict the Intracellular Growth of *Legionella*

(A) DCs or BMMs were fixed either 2 or 12 hr after infection with wild-type *Legionella* producing GFP (green). DCs were stained with an antibody specific for MHC II (red), and BMMs were stained with propidium iodide to label DNA (red). Confocal micrographs show that, at 2 hr, intracellular *Legionella* were detected inside both DCs and BMMs (arrows). At 12 hr, *Legionella* inside of DCs had not replicated (arrow), whereas *Legionella* inside of BMMs had undergone several rounds of multiplication (arrow). Bar = 5 μm.

(B) The efficiency of replicative phagosome formation was determined for *Legionella* after internalization by either DCs or BMMs.

(C) The viability of DCs was monitored using an MTT reduction assay. Values plotted are the OD_{595} measurements obtained at the time point indicated on the *x* axis divided by the OD_{570} measurements obtained prior to infection. A value of <1 indicates a drop in DC viability. Values obtained for uninfected DCs (circles) are compared to values obtained for DCs infected with either wild-type *Legionella* (squares) or *dotA* mutant bacteria (diamonds). Data for each time point are the average of values obtained from three independent wells.

(D) Bone marrow-derived DCs were infected with either wild-type (squares) or *dotA* mutant (diamonds) *Legionella*. Spleen DCs were infected with wild-type *Legionella* (closed circles). CFUs were calculated at the indicated time points after infection to determine the total number of *Legionella* in individual wells. In parallel, the same *Legionella* strains were assayed for their ability to grow in BMMs.

Results

DCs Restrict Intracellular Growth of *Legionella*

Because DCs are likely to play an important role in the initiation of adaptive immunity to vacuolar pathogens, experiments to investigate *Legionella* infection of bone marrow-derived DCs were conducted. DCs containing intracellular *Legionella* were identified by confocal microscopy 2 hr after infection (Figure 1A). Fluorescence microscopy was used to determine whether intracellular *Legionella* could replicate in DCs. Surprisingly, when DCs infected with wild-type *Legionella* were examined 12 hr after infection, large replicative vacuoles were not detected. Most 12 hr phagosomes contained only a single bacterium (Figure 1A). In 2.6% of the DCs infected with wild-type *Legionella*, small vacuoles containing four to six bacteria were scored as replicative vacuoles; however, given the normal 2 hr doubling time for *Legionella* intracellularly, bacterial numbers inside of these organelles were considered low (Figure 1B). By contrast, 75% of the 12 hr phagosomes in infected BMMs contained 16–32 bacteria (Figures 1A and 1B). There were no replicative vacuoles found in BMMs or DCs after infection with an avirulent *dotA* mutant strain of *Legionella* (data not shown). No measurable effect on DC viability was observed over a 72 hr period following infection by *Legionella* (Figure 1C), indicating that virulent *Legionella* were not killing DCs before intracellular bacteria had an opportunity to replicate. The intracellular growth rate of *Legionella* in DCs was compared to BMMs by measuring colony-forming units (CFUs) of bacteria over a period of 48 hr following infection (Figure 1D). There was no measurable increase in bacterial CFUs over 48 hr following infection of DCs (Figure 1D). Similar to results using bone marrow-derived DCs, *Legionella* did not replicate in primary DCs isolated from the spleen (Figure 1D).

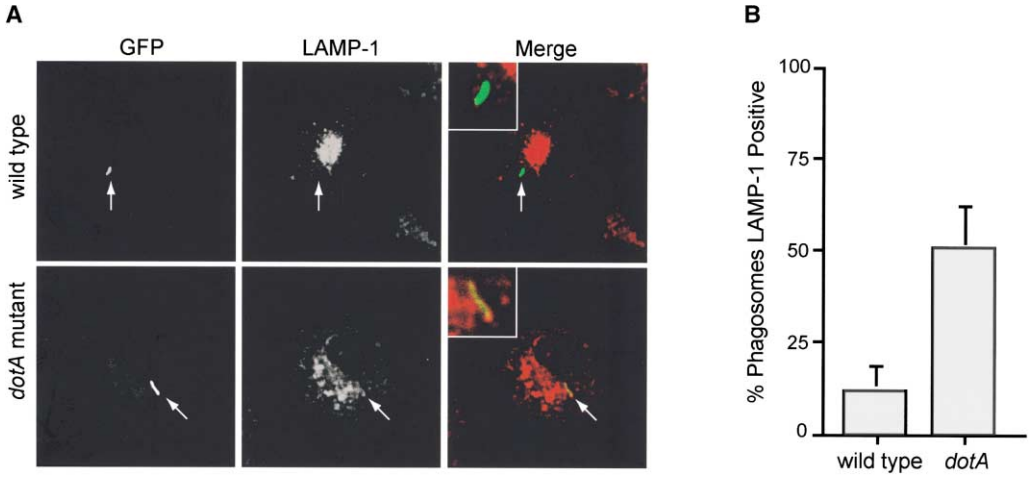

Figure 2. Phagosomes Containing Wild-Type *Legionella* Evade Endocytic Maturation in DCs

(A) DCs were infected with wild-type or *dotA* mutant strains of *Legionella* producing GFP (green) and fixed 1 hr after infection. LAMP-1 staining (red) was used to identify late endosomes and lysosomes. In the upper panels, arrows indicate the location of a wild-type bacterium in a phagosome that does not stain positive for LAMP-1. In the lower panels, arrows indicate the location of a *dotA* mutant residing in a phagosome that is LAMP-1 positive. The insert in each merge panel is a higher magnification of the phagosome indicated by the arrow.

(B) Phagosomes containing either wild-type or *dotA* mutants of *Legionella* were scored for LAMP-1 staining. Shown are the percent of phagosomes containing wild-type *Legionella* that stained positive for LAMP-1 and the percent of phagosomes containing *dotA* mutant bacteria that were LAMP-1 positive. Values are the average ±SD of three independent experiments in which at least 50 phagosomes were scored for each strain.

Using BMMs infected in parallel, exponential growth of wild-type *Legionella* was observed (Figure 1D), demonstrating that these bacteria were competent for intracellular growth. These data independently confirm single-cell assays showing that DCs restrict the intracellular growth of wild-type *Legionella*.

Phagosomes Containing *Legionella* Are Transported to the ER in DCs

Following internalization by macrophages, phagosomes containing *Legionella* evade rapid endocytic maturation and are converted into ER-derived vacuoles (Horwitz, 1983a; Kagan and Roy, 2002; Swanson and Isberg, 1995; Tilney et al., 2001). It is within these ER-derived organelles that *Legionella* begin replicating (Horwitz, 1983a; Horwitz and Silverstein, 1980). Transport of phagosomes containing *Legionella* was examined to determine whether DCs disrupt either evasion of phagosome lysosome fusion or the formation of an ER-derived vacuole. Phagosomal acquisition of the late endosome/lysosome marker LAMP-1 was examined to determine whether phagosomes containing *Legionella* avoid endocytic maturation in DCs. The majority of phagosomes containing wild-type *Legionella* failed to acquire LAMP-1 following infection of DCs (Figure 2), consistent with data using BMMs (Roy et al., 1998). As expected, the majority of phagosomes containing *dotA* mutants of *Legionella* were LAMP-1 positive at this time (Figure 2). Thus, after being internalized by DCs, the phagosomes containing wild-type *Legionella* evade fusion with late endocytic organelles.

Electron microscopy (EM) was used to examine whether phagosomes containing wild-type *Legionella* were converted into ER-derived organelles inside DCs. When DCs infected with wild-type *Legionella* were examined, phagosomes containing *Legionella* were morphologically similar to the ER-derived organelles *Legionella* create in BMMs. ER vesicles, ribosomes, and mitochondria were associated intimately with the membranes of vacuoles containing wild-type *Legionella* (Figure 3A). The membrane surrounding phagosomes containing *dotA* mutants appeared naked, lacking attached vesicles, ribosomes, and mitochondria (Figure 3B). ER transport was also investigated by measuring colocalization of calnexin, a resident ER protein, on *Legionella*-containing phagosomes in DCs at 6 hr postinfection. Roughly 50% (29 of 59) of the phagosomes containing wild-type *Legionella* (Figure 3C) were surrounded by a clear ring of calnexin, whereas calnexin staining of phagosomes containing *dotA* mutants (Figure 3D) was not seen (0 of 54). The proportion of calnexin-positive phagosomes observed in DCs at 6 hr is similar to the proportion of calnexin-positive phagosomes detected at this same time in BMMs (Kagan and Roy, 2002). These data indicate that phagosomes containing *Legionella* traffic to the ER in DCs. Thus, DCs must have a mechanism that restricts bacterial replication after *Legionella* have created a specialized ER-derived organelle.

De Novo Synthesis of *Legionella* Proteins Occurs after DC Uptake

Inhibition of bacterial protein synthesis could be a mechanism by which DCs prevent growth of *Legionella* that are contained in ER-derived organelles. To investigate whether *Legionella* are able to synthesize new proteins after DC internalization, we analyzed bacterial production of a plasmid-encoded GFP reporter that is tightly regulated by an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. When bacterial synthesis of GFP was induced prior to DC infection and maintained

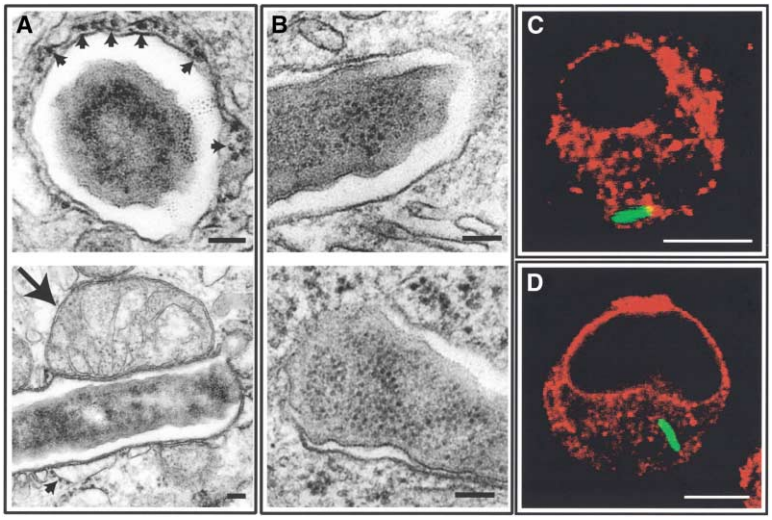

Figure 3. Phagosomes Containing Wild-Type *Legionella* Associate with ER and Mitochondria in DCs

(A) EM micrographs of DCs infected with wild-type *Legionella* show associated ribosomes and ER vesicles (small arrows) and mitochondria (large arrow) surrounding vacuoles harboring *Legionella*. Bar = 0.1 μm.

(B) EM microphages of DCs infected with *dotA* mutant *Legionella* show phagosomes that are devoid of attached vesicles, ribosomes, and mitochondria. Bar = 0.1 μm.

(C) Confocal fluorescent micrograph shows a 6 hr phagosome containing wild-type *Legionella* (green) surrounded by calnexin (red). Bar = 5.0 μm.

(D) Confocal fluorescent micrograph shows no calnexin (red) around a 6 hr phagosome containing a *Legionella dotA* mutant (green). Bar = 5.0 μm.

after infection, both intracellular wild-type and *dotA* mutant bacteria exhibited GFP fluorescence (Figure 4, $IPTG^{+/+}$). Intracellular *Legionella* could not be detected by GFP fluorescence when bacterial GFP expression was not induced before and during DC infection (Figure 4, $IPTG^{-/-}$). However, wild-type *Legionella* were detected by GFP fluorescence when IPTG was added to the tissue culture medium after infection by nonfluorescent bacteria (Figure 4, wild-type, $IPTG^{-/+}$ panels). By contrast, *dotA* mutants remained nonfluorescent after the addition of IPTG to the culture medium (Figure 4, *dotA* mutant, $IPTG^{-/+}$ panels), indicating that de novo synthesis of GFP is restricted inside of the endocytic organelle containing *dotA* mutants. These data demonstrate that *Legionella* remain metabolically active inside of the ER-derived vacuole they create in DCs.

Infection of DCs by *Legionella* Stimulates the Production of IL-12

Bacterial products such as LPS will stimulate IL-12 production by DCs. To investigate whether DCs respond to wild-type *Legionella* and avirulent *dotA* mutants similarly, secretion of the IL-12 p40 subunit by DCs was measured following infection. Secreted IL-12 p40 was not detected in supernatants from uninfected DCs (Table 1). Robust secretion of IL-12 p40 was detected after DCs were infected with either wild-type *Legionella* or *dotA* mutant bacteria. The magnitude of the IL-12 p40 response by DCs following infection with wild-type *Legionella* was greater than that observed when DCs were infected with nonpathogenic *dotA* mutants. In addition to the p40 subunit, the bioactive IL-12 p70 protein was also detected in supernatants from *Legionella*-infected DCs. When BMMs were infected with either wild-type or *dotA* mutants of *Legionella* an IL-12 p40 response was observed that was similar to that detected for DCs (Table 1). These data indicate that *Legionella* infection of DCs triggers IL-12 production.

Table 1. IL-12 Is Produced by APCs upon *L. pneumophila* Infection

APCs	*L. pneumophila*	IL-12p40 pg/ml ± SD	IL-12p70 pg/ml ± SD
DC	wild-type	8591 ± 942	287 ± 48
DC	*dotA*	3924 ± 429	171 ± 62
DC	uninfected	<15[a]	<62[b]
BMM	wild-type	2705 ± 120	ND[c]
BMM	*dotA*	1772 ± 64	ND[c]
BMM	uninfected	282 ± 23	ND[c]

[a]The lower limit of detection for the IL-12 p40 assay was 15 pg/ml.
[b]The lower limit of detection for the IL-12 p70 assay was 62 pg/ml.
[c]ND, not determined.

DCs Infected with *Legionella* Can Process and Present Exogenous Antigens

To prime an adaptive TH-1 type immune response specific for bacterial-derived peptides, DCs must be able to internalize extracellular antigens for processing and presentation on MHC II. To determine whether DCs infected with *Legionella* are able to present foreign antigens on MHC II, we investigated whether the ability of DCs to process hen egg lysozyme (HEL) protein and present the $HEL_{46\text{-}61}$ peptide on MHC II $I\text{-}A^k$ was affected by *Legionella*. DCs infected with either wild-type or *dotA* mutant *Legionella* were incubated in medium containing soluble HEL and double stained with the Rivoli antibody that is specific for a conserved cytoplasmic epitope on all I-A β subunits (Pierre et al., 1997) and with the C4H3 antibody that specifically recognizes the $HEL_{46\text{-}61}$ peptide bound to the $I\text{-}A^k$ molecule (Zhong et al., 1997). Presentation of the $I\text{-}A^k$::$HEL_{46\text{-}61}$ complex was measured as the ratio of C4H3 staining to total MHC II staining in DCs that were infected with *Legionella*. The intensity of C4H3 staining did not differ significantly when cells infected with either wild-type *Legionella* or *dotA* mutants were compared to the uninfected controls (Figure 5). These results indicate that DCs infected with wild-type or *dotA* strains of *Legionella* are fully capable of processing exogenous antigens for presentation on MHC II.

ER Transport Does Not Effectively Sequester *Legionella* Antigens from MHC II Presentation

Even though DCs restrict the growth of wild-type *Legionella*, our data indicate that these bacteria are still

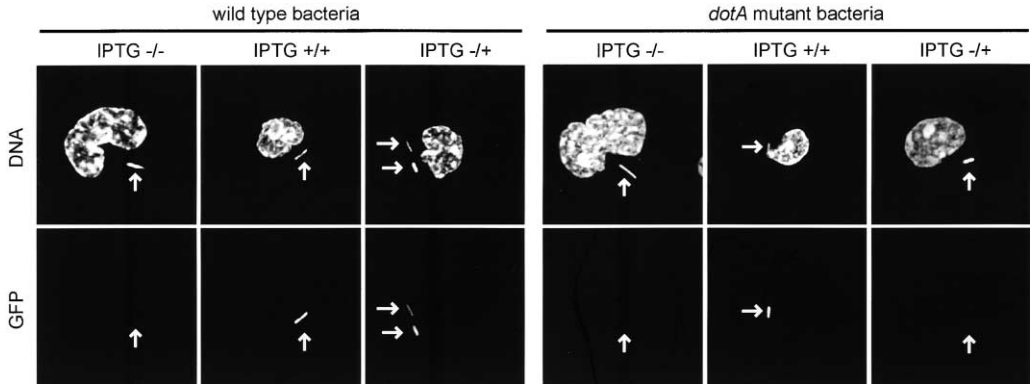

Figure 4. Wild-Type *Legionella* Is Metabolically Active in DCs

DCs were infected with wild-type or *dotA* mutant strains of *Legionella* that contained a plasmid with an IPTG-inducible gene encoding GFP. Cells were stained with propidium iodide after infection to fluorescently label DNA. $IPTG^{+/+}$ indicates that GFP production was induced both prior to infection and during infection. $IPTG^{-/+}$ indicates that GFP production was not induced prior to infection but IPTG was added to the DC medium after infection to induce GFP production intracellularly. $IPTG^{-/-}$ indicates that GFP production was not induced either before or during infection. In the top panel are confocal images of DNA staining that identify the nucleus of the DC and all intracellular bacteria (arrows). Matched images in the bottom panel show only the GFP fluorescence. The arrows in the bottom panels correspond to the location of bacteria shown in the top panel. Images of wild-type bacteria in the $IPTG^{-/+}$ column show that GFP production can be induced after *Legionella* have been internalized by DCs.

able to avoid being delivered to lysosomes and establish residence in an ER-derived vacuole. By residing in an ER-derived vacuole, the repertoire of bacterial antigens available for processing and presentation on MHC II should be limited, which may enable *Legionella* inside of DCs to avoid recognition by antigen-specific T cells. To test this hypothesis, presentation of *Legionella* antigens by infected DCs was measured. Dendritic cells were infected with either wild-type or *dotA* mutants of *Legionella*, and then CD4 T cells isolated from mice immunized with wild-type *Legionella* or from unimmunized control mice were added to infected DCs (Figure 6A). Production of IFN-γ was used to measure antigen-specific TH-1-type T cell responses. When DCs infected

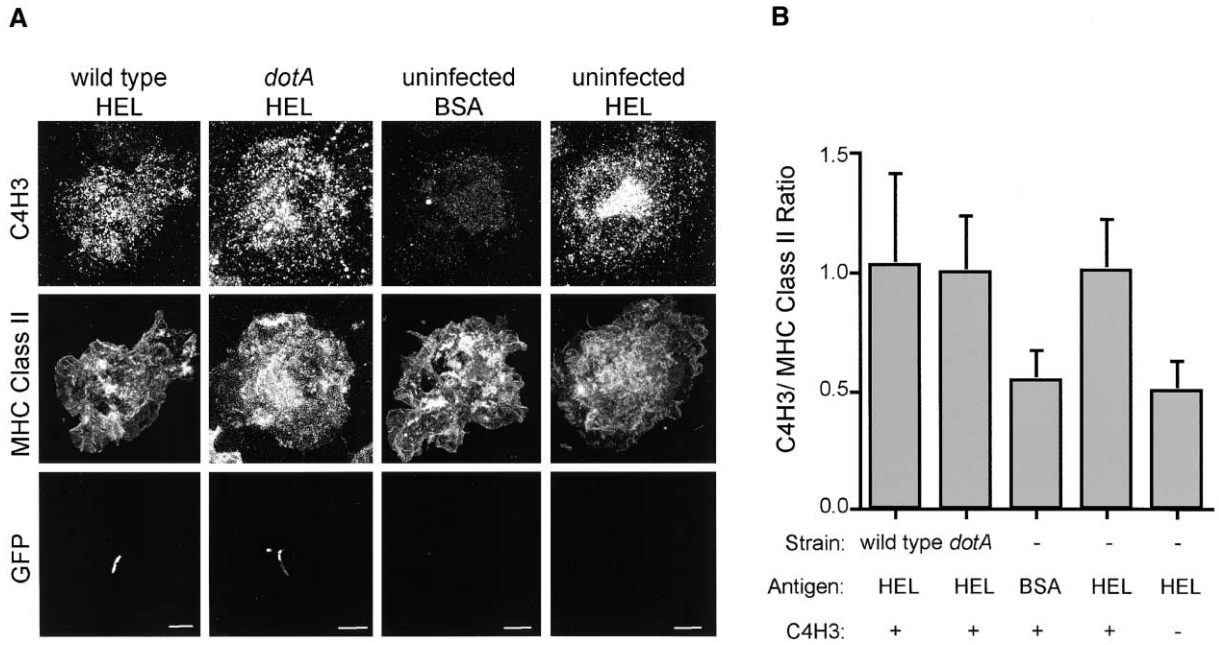

Figure 5. *Legionella* Infection Does Not Inhibit MHC II Presentation by DCs

DCs were infected with either wild-type or *dotA* mutant *Legionella* producing GFP and then pulsed with either soluble HEL or BSA.

(A) Images in each row are separated fluorescent channels from a stacked confocal Z series showing staining of the $I\text{-}A^{k}$::$HEL_{46\text{-}61}$ complex (C4H3), total I-A staining (MHC class II), and bacterial fluorescence (GFP).

(B) Cellular C4H3 staining in DCs is presented as the relative ratio of C4H3 fluorescence to MHC II fluorescence detected in a stacked confocal Z series. Data points are the average ratios $\pm$SD obtained from 20 different projected Z stacks.

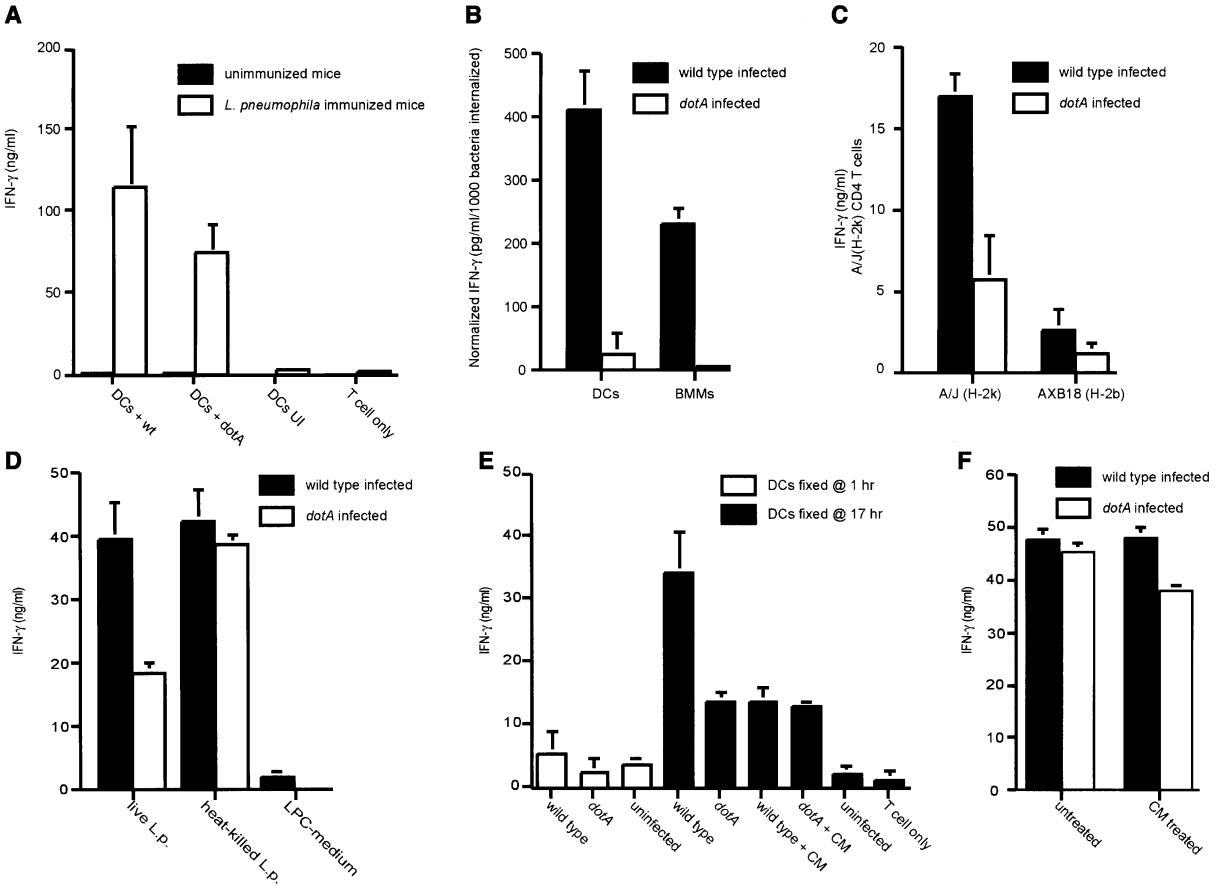

Figure 6. Antigen-Specific CD4 T Cell Recognition of *Legionella*-Infected APCs

(A) $CD4^+$ T cells were isolated from either mice immunized with wild-type *Legionella* (white bars) or unimmunized control mice (black bars). These T cells were coincubated with DCs infected with wild-type *Legionella* (DCs + wt), DCs infected with *dotA* mutant *Legionella* (DCs + *dotA*), uninfected DCs (DCs UI), or in the absence of DCs (T cell only). T cell responses were measured by determining the amount of IFN-γ present in the tissue culture supernatant at 48 hr.

(B) DCs and BMMs were fixed in PFA 17 hr after being infected by either wild-type (black bars) or *dotA* mutant (white bars) *Legionella*. Fixed DCs were incubated with $CD4^+$ T cells from mice immunized with wild-type *Legionella*, and IFN-γ production was measured after 48 hr. IFN-γ production was normalized to bacterial internalization frequencies to compensate for the enhanced efficiency of uptake by BMMs.

(C) DCs from either A/J mice or AXB18 mice were fixed in PFA 17 hr after being infected by either wild-type (black bars) or *dotA* mutant (white bars) *Legionella*. Fixed DCs were incubated with $CD4^+$ T cells from A/J mice immunized with wild-type *Legionella*, and IFN-γ production was measured after 48 hr.

(D) DCs were incubated with either live *Legionella* (live L.p.), heat-killed *Legionella* (heat-killed L.p.), or conditioned tissue culture medium incubated previously with live *Legionella* (LPC-medium). DCs were fixed in PFA 17 hr after being incubated in the medium containing or conditioned by either wild-type (black bars) or *dotA* mutant (white bars) *Legionella*. Fixed DCs were incubated with $CD4^+$ T cells from A/J mice immunized with wild-type *Legionella*, and IFN-γ production was measured after 48 hr.

(E) DCs were fixed in PFA either 1 hr (white bars) or 17 hr (black bars) after being infected by either wild-type or *dotA* mutant *Legionella*. Assays in which chloramphenicol was added to the tissue culture medium following *Legionella* infection to inhibit bacterial protein synthesis are indicated (+ CM). Fixed DCs were incubated with $CD4^+$ T cells from mice immunized with wild-type *Legionella*, and IFN-γ production was measured after 48 hr. Control assays using uninfected DCs or where DCs were not added (T cell only) are indicated.

(F) DCs were fixed in PFA 17 hr after being infected by either wild-type (black bars) or *dotA* mutant (white bars) *Legionella*. Assays in which chloramphenicol was added to the tissue culture medium following *Legionella* infection to inhibit bacterial protein synthesis are indicated (CM treated). Fixed DCs were incubated with $CD4^+$ T cells from A/J mice immunized with *dotA* mutant *Legionella* and IFN-γ production was measured after 48 hr. In each panel, values are the average levels of IFN-γ ±SD from a presentation assay performed in triplicate.

with *Legionella* were used as presenters, T cells from immunized mice responded and produced high levels of IFN-γ. Interestingly, the magnitude of the IFN-γ response was greater for DCs infected with wild-type *Legionella* than for DCs infected with *dotA* mutants. This enhanced T cell response to APCs infected with wild-type *Legionella* was also observed using BMMs (Figure 6B). Because uptake into BMMs is more efficient than into DCs, the T cell responses in this experiment were normalized to the number of *Legionella* internalized. T cells from immunized A/J (H-2k) mice responded poorly to infected DCs derived from AXB18 (H-2b) mice, indicating that IFN-γ production is MHC II restricted (Figure 6C). T cells did not respond to DCs that were incubated in conditioned tissue culture medium containing proteins that might be released by extracellular *Legionella* prior to uptake, indicating that the presented antigens are derived from internalized bacteria as op-

posed to any bacterial debris that was released into the medium during infection and internalized (Figure 6D). These data demonstrate that *Legionella* antigens are efficiently presented on MHC II following infection of APCs and suggest that the ability to reside in an ER-derived vacuole does not effectively prevent presentation of these determinants.

Enhanced Recognition of Infected APCs Requires Selective T Cell Priming and De Novo Synthesis of *Legionella* Proteins Intracellularly

The kinetics of antigen processing and presentation following uptake of *Legionella* by DCs was examined. Using cells fixed 1 hr after infection, the magnitude of the T cell response did not differ significantly for DCs infected with wild-type *Legionella* compared to DCs infected with *dotA* mutants (Figure 6E). By contrast, using cells fixed 17 hr after infection, the T cell response detected for DCs infected with wild-type *Legionella* was more than double that detected for DCs infected with *dotA* mutants (Figure 6E). To determine whether this difference was dependent on de novo synthesis of proteins by intracellular bacteria, the antibiotic chloramphenicol, which will inhibit bacterial but not host protein synthesis, was added to the DC culture after *Legionella* internalization. The addition of chloramphenicol after infection of DCs by *dotA* mutant bacteria did not affect the T cell response. However, for DCs fixed 17 hr after infection with wild-type *Legionella*, the addition of chloramphenicol diminished the T cell response to a level equivalent to that detected using DCs infected with *dotA* mutant bacteria. T cells responded equally to DCs infected with heat-killed wild-type *Legionella* as compared to heat-killed *dotA* mutant bacteria (Figure 6D), further indicating that de novo synthesis of bacterial proteins intracellularly accounts for the enhanced T cell response observed for APCs infected with wild-type *Legionella*.

These data suggest that immunization of mice with wild-type *Legionella* results in the generation of two different categories of T cells: those that can respond to common determinants presented by both wild-type and *dotA* mutant *Legionella* and a subset of T cells that respond to newly synthesized antigens produced only by *Legionella* residing in an ER-derived organelle. This hypothesis was tested using T cells isolated from mice immunized with *dotA* mutant bacteria. As shown (Figure 6F), programmed CD4 T cells from *dotA*-immunized mice gave an equivalent response to DCs infected with wild-type as they did to DCs infected with *dotA* mutant *Legionella*. Additionally, this response was not affected by inhibiting bacterial protein synthesis with chloramphenicol after uptake. These data suggest that mice immunized with a *dotA* mutant fail to produce effector T cells that respond to bacterial determinants synthesized after APCs have been infected with wild-type *Legionella*.

Discussion

Pathogens that infect professional APCs and replicate within nondegradative vacuoles present a unique challenge to the host immune system. Unlike bacteria that are transported to lysosomes and degraded, there is evidence that vacuolar pathogens can sequester antigens to prevent presentation on MHC II (Pancholi et al., 1993). In an effort to understand how adaptive immune responses can then be generated against vacuolar pathogens, we discovered that murine DCs have the ability to restrict intracellular replication of *Legionella*.

This raises the question of whether the ability of DCs to restrict microbial growth may play an important role in immunity to vacuolar pathogens. It is clear that rapid intracellular replication of *Legionella* can kill a naive macrophage within 24 hr (Horwitz and Silverstein, 1980). Thus, it seems unlikely that infected macrophages would be capable of migrating from the lung to lymphoid organs and interact productively with naive CD4 T cells to generate antigen-specific TH-1-type effectors. By contrast, the ability of DCs to survive their encounter with *Legionella* by restricting intracellular growth may have important consequences on the ability of these APCs to stimulate an adaptive immune response. Presumably, *Legionella*-infected DCs will have time to migrate from the site of infection to lymphoid organs and present captured antigens to naive T cells that maintain residence within these tissues, which would facilitate the priming of adaptive immune responses. Following infection by *M. tuberculosis*, or another intracellular pathogen that replicates slowly, DC-mediated growth restriction may not be as important for T cell priming as these APCs could theoretically survive infection long enough to accomplish this task. Consistent with this hypothesis, it has been shown that murine DCs support the intracellular growth of *M. tuberculosis* (Bodnar et al., 2001; Gonzalez-Juarrero and Orme, 2001).

After *Legionella* are internalized by DCs, their phagosomes avoid fusion with endocytic vesicles enriched for LAMP-1 and are converted into ER-derived organelles that are morphologically similar to those formed in macrophages. *Legionella* residing in these ER-derived vacuoles synthesize GFP, meaning that these bacteria remain metabolically active after being internalized by DCs. These data indicate that the mechanism by which DCs restrict replication of *Legionella* allows production of proteins that are induced intracellularly. In addition, cellular events that lead to the selection of *Legionella* antigens for presentation on the surface of infected DCs should be similar to those occurring in infected macrophages. Thus, the repertoire of bacterial determinants being presented by infected DCs should be similar to that on infected macrophages. This means that the naive T cells being stimulated by infected DCs will be specific for many of the same bacterial epitopes being displayed by infected macrophages. This phenomenon may allow selection of a T cell population that will provide the help necessary to activate infected macrophages enabling them to kill intracellular bacteria and resist reinfection.

In support of this model, we found that T cells isolated from mice immunized with wild-type *Legionella* responded better to APCs infected with wild-type *Legionella* compared to APCs infected with *dotA* mutants. These data indicate that wild-type *Legionella* are unable to avoid MHC II presentation by residing in an ER-derived vacuole and suggest that the adaptive response being generated in infected animals may include T cells that are able to recognize bacterial determinants that

are only presented by APCs containing *Legionella* that have established this unique organelle. Additional studies showed that the enhanced T cell response required de novo synthesis of bacterial proteins following internalization of wild-type *Legionella* by DCs and that this enhanced response is not found for T cells programmed during infection by *dotA* mutant bacteria. Thus, there appears to be a subset of T cells primed during infection that are specific for bacterial determinants synthesized and presented after wild-type *Legionella* establish residence in an ER-derived vacuole.

There are several pathways that could account for the delivery of *Legionella* proteins from the ER-derived vacuole into endocytic organelles that mediate antigen processing and presentation. These bacterial proteins could be secreted by *Legionella* into the lumen of the ER-derived vacuole and then be transported in secretory vesicles to endocytic organelles via normal biosynthetic pathways. The recent report of direct membrane fusion between the ER and phagosomes (Gagnon et al., 2002) might also account for the delivery of ER-localized antigens into endocytic compartments. Additionally, it has been reported that ER-derived vacuoles containing *Legionella* sometimes fuse with endocytic organelles during the late stages of macrophage infection (Sturgill-Koszycki and Swanson, 2000), which could result in presentation of antigens in the lumen of the vacuole as well as antigens associated with intact bacteria. Less likely is the idea that *Legionella* antigens are loaded directly onto nascent MHC II in the ER lumen.

In conclusion, these data show that by residing in a vacuole analogous to that found in BMMs, *Legionella* antigens that are processed and presented by DCs reflect the repertoire of antigens presented on the surface of infected BMMs. It is possible that this ability of DCs to restrict intracellular growth of a *Legionella* without altering their transport could be a general property that increases the effectiveness of DCs to prime adaptive immune responses upon microbial infection. This property would enable DCs to stimulate naive T cells that are specific for a subset of antigens displayed specifically on host cells containing replicating bacteria, which are the intended targets of TH-1-directed immune responses to these pathogens.

Experimental Procedures

Bacterial Cultures

The *Legionella serogroup* 1 strain, Lp01 (Berger and Isberg, 1993), and the isogenic *dotA* mutant strain, CR58 (Zuckman et al., 1999), were cultured on charcoal yeast extract (CYE) agar (Feeley et al., 1979) for 2 days prior to use in experiments. GFP-producing *Legionella* harbored the plasmid pAM239 (Coers et al., 1999). In experiments requiring production of GFP protein before infection, *Legionella* were grown on CYE plates supplemented with chloramphenicol (6.25 μg/ml) and IPTG (0.1 mM). For experiments examining GFP fluorescence after infection, *Legionella* was grown on plates supplemented with chloramphenicol only, and GFP expression was induced after infection by adding IPTG (0.2 mM) to the tissue culture medium.

Macrophage and Dendritic Cell Cultures

Unless otherwise indicated, all the BMMs and DCs used in these studies were derived from A/J mice (Jackson Laboratories). Cultures of BMMs were prepared by the method of Celada et al. (1984). Bone marrow-derived DCs were prepared by the method of Inaba et al. (1992) for use in single-cell assays and IL-12 assays. For antigen presentation experiments, DCs were prepared according to the protocol of Lutz et al. (1999), which generates a higher yield of DCs. Spleen DCs were isolated as described (Iwasaki and Kelsall, 1999).

Single-Cell Assays to Measure *Legionella* Uptake and Formation of Replicative Vacuoles

DCs (1×10^6) were added to 24-well tissue culture plates and infected with *Legionella* expressing GFP at a multiplicity of infection (MOI) of 50. Plates were incubated for 2 hr. To remove extracellular bacteria, cells were washed 3× with phosphate-buffered saline (PBS). Cells were fixed either 2 hr or 12 hr postinfection. To fix and stain DCs for microscopy, cells were washed in serum-free medium, added to tissue culture wells containing 12 mm glass coverslips that had been treated in a 1% solution of alcian blue (Sigma) in serum-free medium, incubated for 30 min at 37°C, and then fixed in PBS containing 3.7% paraformaldehyde (PFA) for 20 min at room temperature. After fixation the coverslips were washed 3× with PBS. Coverslips were permeabilized and blocked in PBS containing 2% goat serum and 0.05% saponin (perm-block solution) for 15 min at room temperature. Coverslips were then incubated overnight at 4°C in perm-block containing the Rivoli antibody specific for a conserved cytoplasmic epitope found on all MHC II I-A β subunits (Pierre et al., 1997). Coverslips were washed 3× in PBS containing 0.05% saponin. Coverslips were incubated 1 hr at 37°C with Texas-red-labeled goat anti-rabbit secondary (Zymed) in perm-block solution and then washed 3× with PBS. Coverslips were mounted on slides and examined by fluorescence microscopy. Rivoli staining of MHC II was used to positively identify DCs under the microscope. Assays to measure uptake and formation of *Legionella*-containing vacuoles in BMMs were conducted similarly (Kagan and Roy, 2002). The efficiency of replicative phagosome formation was determined by dividing the percent of host cells containing replicative vacuoles at 12 hr by the percent of cells containing intracellular *Legionella* 2 hr after infection. At least 500 host cells were counted at each time point for each independent assay. Results are from a single assay. Each assay was repeated three times, and the results differed by less than 10%.

Dendritic Cell Viability Assay

DC viability following *Legionella* infection was monitored by measuring 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) reduction as described (Marra et al., 1990). In brief, DCs (1×10^5) were infected with *Legionella* at an MOI of 10 for 2 hr, and then extracellular bacteria were removed by washing the cells 3× with PBS. At 24 hr intervals, MTT was added to representative wells, and the amount of reduced MTT was determined by measuring light absorbance at 570 nm. Measurements made after infection were divided by the measurements taken prior to infection, and these values were plotted. All data points represent the average values ±SD obtained from three wells assayed independently.

Intracellular Growth Curves

Growth of *Legionella* in BMMs was measured as described previously (Zuckman et al., 1999) and modified slightly for DCs. After *Legionella* were added to DC cultures at an MOI of 10, the plates were centrifuged at 150 g for 5 min and then incubated at 37°C for 30 min. Cells were removed from the wells, and DCs were positively selected on magnetic columns using anti-CD11c-coated magnetic beads (Miltenyi Biotech). To remove extracellular bacteria, the DCs were washed 3× with PBS while bound to the column. DCs were eluted and 1×10^5 DCs were added to individual wells in a 96-well round-bottom plate. Lysates from individual wells were prepared at the times indicated after infection and plated on CYE agar to determine bacterial CFUs. Data are the mean CFUs recovered from three independent wells ±SD. It should be noted that the culturing of BMMs in DC medium did not affect intracellular growth of *Legionella* (data not shown).

Phagosome Transport Assays

To examine LAMP-1 acquisition by phagosomes containing *Legionella*, 1×10^6 DCs were infected for 5 min at an MOI of 100, extracellular bacteria were removed by washing 3× in PBS, and cells were

further incubated for 55 min in fresh DC medium. DCs were attached to coverslips and fixed as described above. DCs were stained for LAMP-1 using antibody 1D4B (Chen et al., 1985), and MHC II was stained using the Rivoli antibody. Phagosomes were scored as LAMP-1 positive if an unambiguous ring of LAMP-1 staining was seen around a bacterium. Data shown are the average ±SD of three independent experiments where at least 50 phagosomes were scored.

To examine the formation of ER-derived vacuoles, DCs were infected with wild-type and *dotA* strains of *Legionella* for 5 min at an MOI of 100. Extracellular bacteria were removed, and cells were incubated for 2 hr in fresh medium. Cells were fixed for 1 hr at room temperature in 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M cacodylate buffer (pH 7.4), washed 3× in cacodylate buffer, and then postfixed in 1% osmium tetroxide in the same buffer for 1 hr at room temperature. Cells were then washed 3× in 50 mM sodium maleate (pH 5.2) and stained with 2% uranyl acetate in the same buffer for 1 hr at room temperature in the dark. After staining, cells were washed in water and dehydrated in a graded series of ethanol. They were embedded in EMbed 812 resin. Sixty nanometer sections were cut using a Reichert Ultracut E ultramicrotome and collected on formvar- and carbon-coated nickel grids. Sections were stained with 2% uranyl acetate and lead citrate and examined in a Philips Tecnai 12 electron microscope.

To measure calnexin staining of phagosomes containing *Legionella*, DCs were infected for 5 min at an MOI of 100 with *Legionella* producing the GFP protein, and cells were further incubated for 6 hr after extracellular bacteria were removed. Cells were fixed as described above and stained with an antibody specific for calnexin as described previously (Kagan and Roy, 2002). Phagosomes were scored as calnexin-positive if an unambiguous ring of calnexin staining was seen around a bacterium. Data shown are from a single experiment that was repeated once and yielded similar results.

Assaying Intracellular Synthesis of GFP Protein by *Legionella*

De novo synthesis of GFP by intracellular *Legionella* was measured as described previously (Sturgill-Koszycki and Swanson, 2000), with minor modifications. In brief, DCs were infected at an MOI of 50 with wild-type or *dotA* mutant strains of *Legionella* harboring pAM239. In one set of infections, production of GFP protein was induced prior to infection by growing bacteria on CYE plates containing 0.1 mM IPTG. In the other set of infections the bacteria were grown on CYE plates without IPTG to ensure that there would be no production of GFP prior to infection. After infection and a 1.5 hr chase, IPTG (0.2 mM) was added to indicated wells to stimulate the production of the GFP protein by intracellular bacteria. DCs were incubated at 37°C for an additional 4 hr. Cells were fixed, stained, and examined as described above.

Assays to Measure Processing and Presentation of HEL on MHC II

DCs were plated in 24-well tissue culture dishes at a concentration of 1×10^6 cells/well and infected at an MOI of 50 with *Legionella* strains containing pAM239. After infection for 1 hr, DCs were washed to remove extracellular bacteria and incubated for 1 hr in fresh DC medium containing 1 mg/ml of either HEL or BSA. DCs were washed and incubated for an additional 4 hr in antigen-free medium. DCs were fixed as described above and stained with the Rivoli antibody and the rat monoclonal antibody C4H3 (Zhong et al., 1997). The C4H3 antibody specifically binds to the MHC II complex consisting of the $HEL_{46\text{-}61}$ peptide bound to the $I\text{-}A^k$ molecule. After overnight incubation with primary antibodies, coverslips were washed 3× in perm-block and stained with fluorescently labeled secondary antibodies. Confocal Z stacks were acquired for DCs containing *Legionella*, and 20 cells were imaged for each condition. The ratio of C4H3 to Rivoli fluorescence represents the efficiency of MHC II processing and presentation of the soluble HEL protein. Fluorescence ratios obtained for DCs infected with *Legionella* were compared to ratios obtained for uninfected DCs that had been pulsed with either HEL or BSA.

IL-12 Assays

DCs and BMMs were added to 96-well round bottom plates at a concentration of 1×10^5 cells/well. Host cells were infected with *Legionella* at an MOI of 10 or left uninfected. Supernatants were collected at 24 hr and IL-12 production was measured by ELISA using Pharmingen IL-12 p40 and p70 reagents. Data represent the average IL-12 content of three independent wells ±SD.

Assays to Measure Presentation of *Legionella* Antigens

Legionella-specific CD4 T cells were produced by immunizing 6- to 8-week-old A/J mice intraperitoneally (IP) with 1×10^6 CFU of wild-type or *dotA Legionella*, and then boosting 2 weeks after immunization with 1×10^6 CFU of *Legionella*. Spleens were isolated from mice 6 days after boosting, and CD4 T cells were positively selected using the MACS system (Miltenyi Biotech). CD4 T cells from unimmunized mice were isolated in parallel. The individual panels in Figure 6 represent internally controlled assays that used the same population of isolated T cells. T cells from mice immunized independently were used in different panels. Thus, T cell responses should only be compared to internal controls in a given panel. All results shown in Figure 6 were confirmed at least two times with assays done weeks apart with T cells from mice immunized independently.

To measure antigen presentation by live DCs, immature DCs were infected with *Legionella* at an MOI of 10 for 4 hr, washed 3× with PBS to remove extracellular bacteria, and replated into 96-well dishes at a concentration of 1×10^5 cells/well. CD4 T cells (4×10^5) were added to the DCs. Cells were incubated for 48 hr at 37°C in T cell medium (RPMI 1640 containing 10% FBS, 1% MEM nonessential amino acids, 1% MEM amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 10 mM HEPES [pH 7.55], 100 units/ml Penicillin, 100 μg/ml Streptomycin), and then supernatants were collected from each well. T cell responses were measured by determining the amount of IFN-γ present in these culture supernatants using an ELISA assay (Pharmingen). Data represents the average IFN-γ concentration for three independent wells ±SD.

When using fixed DCs, immature DCs were infected with *Legionella* at an MOI of 20 for 1 hr, and then DCs were positively selected on magnetic columns using anti-CD11c magnetic beads (Miltenyi Biotech). After DCs were eluted, they were divided equally into three aliquots. To determine the number of *Legionella* internalized, 1×10^5 DCs from one sample were disrupted by hypotonic lysis, and bacterial CFUs were measured on CYE agar plates. The remaining DCs in this sample were placed immediately into 2% PFA for 20 min at room temperature. Fresh DC medium was added to the remaining samples of infected DCs. To one sample chloramphenicol (12.5 μg/ml) was added, and the other was left untreated. DCs were further incubated for 16 hr and then fixed in 2% PFA for 20 min at room temperature. Fixed DCs (1×10^5) in T cell medium were added to 96-well flat-bottom dishes. CD4 T cells were isolated as described above, and 5×10^5 T cells were added to wells containing fixed DCs. After incubation for 48 hr at 37°C, supernatants were collected and IFN-γ levels were measured by ELISA. Antigen presentation by macrophages was measured similarly.

To obtain heat-killed bacteria, a 1 ml suspension of 1×10^9 *Legionella* was incubated at 80°C for 45 min. Conditioned tissue culture medium containing *Legionella* proteins shed by extracellular bacteria during DC infection was prepared by doing mock infections in wells that did not contain DCs. After 1 hr, intact bacteria were removed from the conditioned medium by centrifugation at 1000 g for 10 min. To compare levels of antigen presentation, DCs were incubated with either bacteria-conditioned medium for 16 hr or with medium containing either live or heat-killed bacteria. DCs were then fixed and T cell responses were measured as described above.

To determine whether T cell responses being measured are MHC II restricted, DCs were prepared from both A/J (H-2k) mice and recombinant inbred AXB18 (H-2b) mice (Beckers et al., 1995). These DCs were infected at an MOI of 20 with wild-type and *dotA* strains of *Legionella* as described above. Cells were fixed at 17 hr postinfection and incubated with CD4 T cells derived from A/J mice immunized with wild-type *Legionella*. After incubation for 48 hr at 37°C, supernatants were collected and IFN-γ levels were measured by ELISA.

Acknowledgments

The authors wish to thank J. Kagan for helpful discussions and critical reading of the manuscript; W. Garrett, A. Chow, A. Iwasaki,

and I. Mellman for technical assistance and reagents; R. Germain for the C4H3 antibody; and M. Pypeart for EM assistance. This work was supported by NIH grant AI48770.

Received: July 10, 2002
Revised: March 13, 2003
Accepted: April 9, 2003
Published: June 17, 2003

References

Albert, M.L., Pearce, S.F., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L., and Bhardwaj, N. (1998). Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. J. Exp. Med. *188*, 1359–1368.

Beckers, M.C., Yoshida, S., Morgan, K., Skamene, E., and Gros, P. (1995). Natural resistance to infection with *Legionella pneumophila*: chromosomal localization of the Lgn1 susceptibility gene. Mamm. Genome *6*, 540–545.

Berger, K.H., and Isberg, R.R. (1993). Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol. Microbiol. *7*, 7–19.

Bodnar, K.A., Serbina, N.V., and Flynn, J.L. (2001). Fate of *Mycobacterium tuberculosis* within murine dendritic cells. Infect. Immun. *69*, 800–809.

Celada, A., Gray, P.W., Rinderknecht, E., and Schreiber, R.D. (1984). Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. J. Exp. Med. *160*, 55–74.

Chen, J.W., Pan, W., D'Souza, M.P., and August, J.T. (1985). Lysosome-associated membrane proteins: characterization of LAMP-1 of macrophage P388 and mouse embryo 3T3 cultured cells. Arch. Biochem. Biophys. *239*, 574–586.

Coers, J., Monahan, C., and Roy, C.R. (1999). Modulation of phagosome biogenesis by *Legionella pneumophila* creates an organelle permissive for intracellular growth. Nat. Cell Biol. *1*, 451–453.

Feeley, J.C., Gibson, R.J., Gorman, G.W., Langford, N.C., Rasheed, J.K., Mackel, D.C., and Blaine, W.B. (1979). Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J. Clin. Microbiol. *10*, 437–441.

Fraser, D.W., Tsai, T.R., Orenstin, W., Parken, W.E., Beechan, H.J., Sharrar, R.G., Harris, J., Mallison, G.F., Martin, S.M., McDade, J.E., et al. (1977). Legionnaires' disease: description of an epidemic of pneumonia. N. Engl. J. Med. *297*, 1189–1197.

Friedman, H., Klein, T.W., Widen, R., Newton, C., Blanchard, D.K., and Yamamoto, Y. (1988). *Legionella pneumophila* immunity and immunomodulation: nature and mechanisms. Adv. Exp. Med. Biol. *239*, 327–341.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., Paiement, J., Bergeron, J.J., and Desjardins, M. (2002). Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell *110*, 119–131.

Gonzalez-Juarrero, M., and Orme, I.M. (2001). Characterization of murine lung dendritic cells infected with *Mycobacterium tuberculosis*. Infect. Immun. *69*, 1127–1133.

Halme, S., and Surcel, H.M. (1997). Cell mediated immunity to *Chlamydia pneumoniae*. Scand. J. Infect. Dis. Suppl. *104*, 18–21.

Heufler, C., Koch, F., Stanzl, U., Topar, G., Wysocka, M., Trinchieri, G., Enk, A., Steinman, R.M., Romani, N., and Schuler, G. (1996). Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur. J. Immunol. *26*, 659–668.

Horwitz, M.A. (1983a). Formation of a novel phagosome by the Legionnaires' disease bacterium (*Legionella pneumophila)* in human monocytes. J. Exp. Med. *158*, 1319–1331.

Horwitz, M.A. (1983b). The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome lysosome fusion in human monocytes. J. Exp. Med. *158*, 2108–2126.

Horwitz, M.A., and Silverstein, S.C. (1980). Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. J. Clin. Invest. *66*, 441–450.

Inaba, K., and Steinman, R.M. (1985). Protein-specific helper T-lymphocyte formation initiated by dendritic cells. Science *229*, 475–479.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J. Exp. Med. *176*, 1693–1702.

Inaba, K., Inaba, M., Naito, M., and Steinman, R.M. (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J. Exp. Med. *178*, 479–488.

Iwasaki, A., and Kelsall, B.L. (1999). Freshly isolated Peyer's patch, but not spleen, dendritic cells produce interleukin 10 and induce the differentiation of T helper type 2 cells. J. Exp. Med. *190*, 229–239.

Kagan, J.C., and Roy, C.R. (2002). *Legionella* phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. Nat. Cell Biol. *4*, 945–954.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77–92.

Macatonia, S.E., Hosken, N.A., Litton, M., Vieira, P., Hsieh, C.S., Culpepper, J.A., Wysocka, M., Trinchieri, G., Murphy, K.M., and O'Garra, A. (1995). Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J. Immunol. *154*, 5071–5079.

Marra, A., Horwitz, M.A., and Shuman, H.A. (1990). The HL-60 model for the interaction of human macrophages with the Legionnaires' disease bacterium. J. Immunol. *144*, 2738–2744.

Mellman, I., Turley, S.J., and Steinman, R.M. (1998). Antigen processing for amateurs and professionals. Trends Cell Biol. *8*, 231–237.

Nagai, H., Kagan, J.C., Zhu, X., Kahn, R.A., and Roy, C.R. (2002). A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science *295*, 679–682.

Orme, I.M., Andersen, P., and Boom, W.H. (1993). T cell response to *Mycobacterium tuberculosis*. J. Infect. Dis. *167*, 1481–1497.

Pancholi, P., Mirza, A., Bhardwaj, N., and Steinman, R.M. (1993). Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science *260*, 984–986.

Pierre, P., Turley, S.J., Gatti, E., Hull, M., Meltzer, J., Mirza, A., Inaba, K., Steinman, R.M., and Mellman, I. (1997). Developmental regulation of MHC class II transport in mouse dendritic cells. Nature *388*, 787–792.

Reis e Sousa, C., Stahl, P.D., and Austyn, J.M. (1993). Phagocytosis of antigens by Langerhans cells in vitro. J. Exp. Med. *178*, 509–519.

Roy, C.R., Berger, K., and Isberg, R.R. (1998). *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. Mol. Microbiol. *28*, 663–674.

Seder, R.A., Gazzinelli, R., Sher, A., and Paul, W.E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. Proc. Natl. Acad. Sci. USA *90*, 10188–10192.

Segal, G., Purcell, M., and Shuman, H.A. (1998). Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA *95*, 1669–1674.

Sinai, A.P., and Joiner, K.A. (1997). Safe haven: the cell biology of nonfusogenic pathogen vacuoles. Annu. Rev. Microbiol. *51*, 415–462.

Steinman, R.M. (1991). The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. *9*, 271–296.

Sturgill-Koszycki, S., and Swanson, M.S. (2000). *Legionella pneumophila* replication vacuoles mature into acidic, endocytic organelles. J. Exp. Med. *192*, 1261–1272.

Swanson, M.S., and Isberg, R.R. (1995). Association of *Legionella*

pneumophila with the macrophage endoplasmic reticulum. Infect. Immun. *63*, 3609–3620.

Tilney, L.G., Harb, O.S., Connelly, P.S., Robinson, C.G., and Roy, C.R. (2001). How the parasitic bacterium *Legionella pneumophila* modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane. J. Cell Sci. *114*, 4637–4650.

Vogel, J.P., Andrews, H.L., Wong, S.K., and Isberg, R.R. (1998). Conjugative transfer by the virulence system of *Legionella pneumophila*. Science *279*, 873–876.

Zhong, G., Reis e Sousa, C., and Germain, R.N. (1997). Production, specificity, and functionality of monoclonal antibodies to specific peptide-major histocompatibility complex class II complexes formed by processing of exogenous protein. Proc. Natl. Acad. Sci. USA *94*, 13856–13861.

Zuckman, D.M., Hung, J.B., and Roy, C.R. (1999). Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. Mol. Microbiol. *32*, 990–1001.