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Dendritic Cells Pulsed with Live and Dead *Legionella pneumophila* Elicit Distinct Immune Responses

Toshiaki Kikuchi, Takao Kobayashi, Kazunori Gomi, Takuji Suzuki, Yutaka Tokue, Akira Watanabe, and Toshihiro Nukiwa

*Legionella pneumophila* is the causative pathogen of Legionnaires’ disease, which is characterized by severe pneumonia. In regard to the pathophysiology of *Legionella* infection, the role of inflammatory phagocytes such as macrophages has been well documented, but the involvement of dendritic cells (DCs) has not been clarified. In this study, we have investigated the immune responses that DCs generate in vitro and in vivo after contact with *L. pneumophila*. Heat- and formalin-killed *L. pneumophila*, but not live *L. pneumophila*, induced immature DCs to undergo similar phenotypic maturation, but the secreted proinflammatory cytokines showed different patterns. The mechanisms of the DC maturation by heat- or formalin-killed *L. pneumophila* depended, at least in part, upon Toll-like receptor 4 signaling or on *Legionella* LPS, respectively. After transfer to naive mice, DCs pulsed with dead *Legionella* produced serum Ig isotype responses specific for *Legionella*, leading to protective immunity against an otherwise lethal respiratory challenge with *L. pneumophila*. The in vivo immune responses required the Ag presentation of DCs, especially that on MHC class II molecules, and the immunity yielded cross-protection between clinical and environmental strains of *L. pneumophila*. Although the DC maturation was impaired by live *Legionella*, macrophages were activated by live as well as dead *L. pneumophila*, as evidenced by the up-regulation of MHC class II. Finally, DCs, but not macrophages, exhibited a proliferative response to live *L. pneumophila* that was consistent with their cell cycle progression. These findings provide a better understanding of the role of DCs in adaptive immunity to *Legionella* infection.


Dendritic cells (DCs) are APCs with an exquisite ability to interact with T cells and modulate their responses for the generation of immunological memory (i.e., Ag-specific adaptive immunity) (5, 6). In contrast to mononuclear phagocytes, the primary functions of which are aimed at the clearance of invading microorganisms by their avid ingestion, which is known as Ag-nonspecific innate immunity, DCs phagocytose pathogens and subsequently migrate to the lymphoid organs, where DCs display Ags processed and loaded on MHC molecules (5–10). During migration, DCs exhibit drastic changes in their features, termed maturation, including the up-regulation of MHC, costimulatory (e.g., B7-1 and B7-2), adhesion (e.g., ICAM-1), and signaling molecules (e.g., CD40) and the induced production of proinflammatory cytokines, resulting in the improved ability of DCs to activate Ag-specific T cells, especially CD4+ Th cells, in a MHC class II-restricted manner (5–10). Thereafter, activated Ag-specific CD4+ T cells stimulate the immune effectors (e.g., Ag-specific B cells) (5–10).

Accordingly, we hypothesized that DCs also play a key role in boosting the adaptive immune responses against *Legionella* infection. To evaluate this hypothesis, we pulsed mouse bone marrow-derived DCs with live or dead *Legionella* and assessed their phenotype changes in vitro and their ability to induce anti-*Legionella* immunity in vivo. The data demonstrate that DCs pulsed with dead *L. pneumophila*, but not DCs pulsed with live *L. pneumophila*, undergo phenotypic maturation and, when adoptively transferred, render mice resistant to a lethal pulmonary infection of *Legionella*. It was also shown that live *L. pneumophila* impairs the maturation of DCs despite its ability to induce the activation of macrophages.

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3. Abbreviations used in this paper: DC, dendritic cell; TLR, Toll-like receptor.
Materials and Methods

Mice
Female C57BL/6 (H-2\(^b\)) and BALB/c mice (H-2\(^d\)) (Charles River Breeding Laboratories, Atsugi, Japan); A/J (H-2\(^a\)), C3H/HeN (H-2\(^k\)), and C3H/HeJ (B6.129-B2m\(^{tm1}\)) (Taconic Farms, Germantown, NY), 6–8 wk old, were housed under pathogen-free conditions until infection.

Bacterial strains
The L. pneumophila strains used in this study included a clinical isolate (referred to as Suzuki strain, serogroup 1) and an environmental isolate (serogroup 5). All Legionella experiments in this study used the Suzuki strain of L. pneumophila, unless otherwise noted. The Esche-richia coli strain 25922 was obtained from American Type Culture Collection (Manassas, VA). L. pneumophila was grown on buffered charcoal yeast extract agar plates or buffer yeast extract broth, as previously published (11). Luria-Bertani broth was used as the growth medium for E. coli.

The bacteria were washed three times and suspended in sterile PBS, pH 7, before use, and the concentration was adjusted spectrophotometrically.

DC and macrophage preparations
DCs were generated from mouse bone marrow precursors in complete RPMI 1640 medium (10% heat-inactivated FBS, 2 mM L-glutamine, 100 \(\mu\)g/ml streptomycin, and 100 U/ml penicillin) with 10 ng/ml mouse rGM-CSF (Peprotech, Rockyford, IL); LPS from Escherichia coli 055:B5 (Sigma-Aldrich, St. Louis, MO) was added at 10 ng/ml to the medium. In some experiments, macrophages were pulsed with L. pneumophila, as described for DCs. For immunization, the pulsed DCs were injected i.v. at 5 x 10\(^6\) cells per mouse. Three weeks after infection, lethal respiratory infection with L. pneumophila was inoculated into the lung. All animals were monitored daily for 14 days after the inoculation. Obviously moribund mice were sacrificed, and this was recorded as the time of death.

Anti-Legionella Abs
Abs against L. pneumophila in sera were assessed by ELISA in microtiter plates (Nalge Nunc International, Rochester, NY) coated with 10\(^7\) CFU gentamicin-killed (50 \(\mu\)g/ml, 1 h) L. pneumophila per well. Serum was serially diluted in TBS (pH 7.4) containing 0.5% BSA for the analysis. Rabbit IgG Abs against mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA were used as secondary Abs for the isotype determination of anti-Legionella Abs, and secondary alkaline phosphatase-conjugated Ab against rabbit IgG was used for the detection (all Abs were from Pierce Biotechnology, Rockford, IL). After the addition of p-nitrophenyl phosphate substrate solution (Pierce Biotechnology), absorbance of the reaction was measured at 405 nm.

Flow cytometric analysis and cytokine ELISA
For DC surface Ag expression, DCs were incubated with FITC- or PE-conjugated mAbs against I-A\(^k\) (MHC class II, clone 11-5.2), CD40 (clone 1G10), CD54 (ICAM-1, clone 3E2), CD80 (B7-1, clone 16-10A1), and CD86 (B7-2, clone GL1), or appropriate isotype-matched control Abs (BD PharMingen, San Jose, CA). For DC proliferation, Flow-Count Fluoro-

spheres (Beckman Coulter, Miami, FL) were used to calibrate the count of the cells. Cells were analyzed on an EPICS XL cytometer with EXP302 ADC software (Beckman Coulter). Dead cells and debris were excluded from the analysis by gating on the appropriate forward scatter, side scatter, and propidium iodide-staining profile. To determine the percentage of stained cells above the isotype control staining, 1% of false positive events was accepted in the control Ab. For cell cycle analysis, DCs were fixed with 70% ethanol, and their DNA was stained with 20 \(\mu\)g/ml propidium iodide. Stained cells were analyzed on the flow cytometer with MultiCycle software (Phoenix Flow Systems, San Diego, CA). The concentrations of specific cytokines released into the medium were measured using ELISA kits for mouse IL-12/40, TNF-\(\alpha\), IL-6, or IL-1\(\beta\) (BioSource International, Camarillo, CA).

Statistical analysis
All data are reported as mean ± SE, unless otherwise noted. Statistical comparison was made using the two-tailed Student’s \(t\) test, and a value of \(p < 0.05\) was accepted as indicating significance. Survival evaluation was conducted using Kaplan-Meier analysis.

Results
Surface phenotype of L. pneumophila-pulsed DCs
Heat- and formalin-killed L. pneumophila induced DC maturation in vitro, but live L. pneumophila did not (Fig. 1). Compared with naïve DCs, the percentages of positive cells for surface markers characteristic of mature DCs, including I-A\(^k\) (MHC class II; Fig. 1A), CD40 (Fig. 1B), CD54 (ICAM-1; Fig. 1C), CD80 (B7-1; Fig. 1D), and CD86 (B7-2; Fig. 1E), were increased. The percentages of stained cells above the isotype control staining were shown in each panel.

FIGURE 1. Flow cytometric analysis of L. pneumophila-pulsed DCs. DCs from A/J mice were pulsed with heat-killed, formalin-killed, or live L. pneumophila at a ratio of 50 bacteria per 1 DC, and analyzed 2 days later for accessory molecule expression by flow cytometry (bold line): A, I-A\(^k\) (MHC class II); B, CD40; C, CD54 (ICAM-1); D, CD80 (B7-1); E, CD86 (B7-2). Overlay histogram (gray, filled) in each panel depicts naïve DCs as a control. The percentages of stained cells above isotype control staining are shown in each panel.

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Cytokines secreted from L. pneumophila-pulsed DCs

ELISA analyses demonstrated that heat- or formalin-killed *L. pneumophila* induced DCs to secrete proinflammatory cytokines with distinct (IL-12 and TNF-α) or similar (IL-6 and IL-1β) intensities, but, with the exception of IL-1β secretion, live *L. pneumophila* did not (Fig. 2). Pulsing of DCs with heat- or formalin-killed *L. pneumophila* resulted in IL-12 induction peaking at 36 h (687 ± 12 pg/ml) or 24 h (301 ± 29 pg/ml), respectively (Fig. 2A). Heat- or formalin-killed *L. pneumophila* also stimulated TNF-α secretion from DCs, and the TNF-α levels were 235 ± 25 or 514 ± 35 pg/ml, respectively, at the peak (heat-killed *Legionella*, 48 h; formalin-killed *Legionella*, 12 h; Fig. 2B). In response to heat- and formalin-killed *L. pneumophila*, IL-6 secretion from DCs reached a similar peak (2964 ± 153 and 2750 ± 259 pg/ml, respectively) at the time point of 24 h and then stayed at about the same level for another 24 h (Fig. 2C). Heat- and formalin-killed *L. pneumophila* caused similarly enhanced secretion of IL-1β from DCs, the highest being 265 ± 22 and 233 ± 19 pg/ml, respectively, at the time point of 2 h (Fig. 2D). IL-1β secretion from DCs was enhanced also by live *L. pneumophila*, and the highest level was 121 ± 16 pg/ml at the time point of 6 h (Fig. 2D). Apart from IL-1β, DCs pulsed with live *L. pneumophila* secreted relatively low levels of cytokines, including IL-12, TNF-α, and IL-6, during the culture period as did naïve DCs (live *Legionella*, IL-12 < 91 pg/ml, TNF-α < 65 pg/ml, IL-6 < 609 pg/ml; naïve DCs, IL-12 < 41 pg/ml, TNF-α < 83 pg/ml, IL-6 < 358 pg/ml, IL-1β < 58 pg/ml; Fig. 2).

**LPS dependency and independency**

To assess whether the DC maturation induced by heat- and formalin-killed *L. pneumophila* depends on LPS, an immunostimulatory component of *L. pneumophila*, we supplemented cultures for bacterial pulsing with polymyxin B, a well-characterized pharmacologic LPS antagonist (17) (Fig. 3, A and B). Compared with DCs pulsed in the absence of polymyxin B, the maturation of DCs pulsed with formalin-killed *L. pneumophila* was suppressed by the presence of polymyxin B in the DC priming, whereas the maturation of DCs pulsed with heat-killed *L. pneumophila* was not adversely affected, as indicated by the percentage of CD86+ cells on flow cytometric analyses (heat-killed *Legionella*, 33.3 vs 32.2%; formalin-killed *Legionella*, 30.1 vs 37.0%; Fig. 3A). In control experiments using *E. coli* LPS, polymyxin B clearly suppressed the LPS-stimulated DC maturation, and the proportion of CD86+ cells markedly decreased (17.2 vs 43.5%; Fig. 3A). Similar results were observed with expression of other surface markers examined, except for CD40 (Table II). No apparent changes were observed in the CD86 expression of naive and live *Legionella*-pulsed DCs, regardless of polymyxin B supplementation (data not shown).

Consistent with this, IL-12 secretion from DCs in response to formalin-killed *L. pneumophila* and *E. coli* LPS was inhibited by the addition of polymyxin B compared with each control (i.e., pulsing without polymyxin B), whereas IL-12 secretion from all other DCs was not affected by polymyxin B supplementation (formalin-killed *Legionella*, p < 0.005; LPS, p < 0.05; naïve DCs, p > 0.7; heat-killed *Legionella*, p > 0.9; live *Legionella*, p > 0.6; Fig. 3B). These data suggest that the DC maturation by formalin-killed *L. pneumophila* depends, at least in part, on *Legionella* LPS, but the DC maturation by heat-killed *L. pneumophila* does not at all.

Table 1. Phenotype changes of DCs pulsed with *L. pneumophila* at different ratios

<table>
<thead>
<tr>
<th>Ratio (Legionella:DC)</th>
<th>Positive Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-A&lt;sup&gt;+&lt;/sup&gt; (MHC class II)</td>
</tr>
<tr>
<td>Naive DCs</td>
<td>31.7</td>
</tr>
<tr>
<td>Heat-killed <em>L. pneumophila</em></td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>43.6</td>
</tr>
<tr>
<td>50:1</td>
<td>48.4</td>
</tr>
<tr>
<td>100:1</td>
<td>49.0</td>
</tr>
<tr>
<td>Formalin-killed <em>L. pneumophila</em></td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>39.8</td>
</tr>
<tr>
<td>50:1</td>
<td>46.8</td>
</tr>
<tr>
<td>100:1</td>
<td>48.9</td>
</tr>
<tr>
<td>Live <em>L. pneumophila</em></td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>21.7</td>
</tr>
<tr>
<td>50:1</td>
<td>0.7</td>
</tr>
<tr>
<td>100:1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The study was similar to that in Fig. 1, but DCs were pulsed with *L. pneumophila* at several ratios of bacteria to DC.
Toll-like receptor 4 (TLR4) signaling dependency and independency

We next investigated whether the DC response to heat- and formalin-killed *L. pneumophila* depends on TLR4 signaling, which has been implicated in the response to *E. coli* LPS, by using TLR4 mutant mice (C3H/HeJ, unresponsive to L. pneumophila). In contrast, when pulsed with formalin-killed *L. pneumophila* from TLR4 mutant mice displayed slightly more CD86+ cells than those from wild-type mice (38.8 vs 36.1%; Fig. 3C). Similar results were observed with CD54 expression (TLR4 mutant vs wild type: heat-killed Legionella, 46.5 vs 53.4%; formalin-killed Legionella, 59.3 vs 51.7%; E. coli LPS, 45.7 vs 57.3%; data not shown). No apparent differences between TLR4 mutant and wild-type DCs were observed in the CD86 expression of naive and live *Legionella*-pulsed DCs (data not shown). This was relevant to the level of IL-12 secretion from TLR4 mutant and wild-type DCs (Fig. 3D). Enhanced secretion of IL-12 from wild-type DCs pulsed with heat-killed *L. pneumophila* and *E. coli* LPS was significantly abrogated in TLR4 mutant DCs (heat-killed *Legionella*, p < 0.05; LPS, p < 0.005), whereas the levels of IL-12 secretion from wild-type and TLR4 mutant DCs were comparable in all other groups (naive DCs, p > 0.3; formalin-killed *Legionella*, p > 0.7; live *Legionella*, p > 0.05; Fig. 3D).

These data indicate that at least one pathway mediated by the TLR4 signaling is involved in the signaling through which heat-killed *L. pneumophila* acts on DCs, but that the TLR4 signaling is not required for the response of DCs to formalin-killed *L. pneumophila* at all. Taken together with our data showing that DC maturation caused by heat- and formalin-killed *L. pneumophila* is independent and dependent, respectively (Fig. 3, A and B), these findings led to the conclusion that the effects of *L. pneumophila* LPS are not mediated by signaling through TLR4.

Anti-*Legionella* Ab responses of immunized mice

The in vivo immune response by DCs pulsed with *L. pneumophila* was assessed by determining the serum level of *Legionella*-specific Abs (Fig. 4). AJ mice immunized with DCs pulsed with heat-killed, formalin-killed, and live *L. pneumophila* produced significant amounts of all serum anti-*Legionella* Ab isotypes examined compared with mice immunized with naive DCs, except for IgA (OD405 at 1:90: IgM, p < 0.01; IgG1, p < 0.05; IgG2a, p < 0.05; IgG2b, p < 0.05; IgG3, p < 0.01; IgA, p > 0.1; Fig. 4). The Ab levels induced by DCs pulsed with heat- and formalin-killed *L. pneumophila* were comparable in almost all isotypes we assayed (OD405 at 1:90: IgM, p < 0.05; IgG1, p > 0.7; IgG2a, p > 0.4; IgG2b, p > 0.8; IgG3, p > 0.3; IgA, p > 0.3), and were higher than those generated by DCs pulsed with live *L. pneumophila*, despite significant differences only in IgM and IgG3 isotypes (OD405 at 1:90: IgM, p < 0.05; IgG1, p > 0.1; IgG2a, p > 0.05; IgG2b, p > 0.4; IgG3, p < 0.05; IgA, p > 0.3; Fig. 4). Similar results were achieved in C57BL/6 mice immunized with *Legionella*-pulsed DCs (data not shown).

In vivo protective effects of *L. pneumophila*-pulsed DCs

The observed difference in anti-*Legionella* levels was relevant to the protection of immunized mice against a lethal bronchopulmonary infection of *L. pneumophila* (Fig. 5, A and B). Immunization of C57BL/6 mice with DCs pulsed with heat- or formalin-killed *L. pneumophila* B1730 DCs and *L. pneumophila*

TABLE II. Surface phenotype of DCs pulsed with *L. pneumophila* in the presence or absence of polymyxin B

<table>
<thead>
<tr>
<th></th>
<th>CD40</th>
<th>CD54</th>
<th>CD80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-killed <em>L. pneumophila</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB−</td>
<td>47.5</td>
<td>61.2</td>
<td>43.7</td>
</tr>
<tr>
<td>PB+</td>
<td>51.6</td>
<td>60.3</td>
<td>52.3</td>
</tr>
<tr>
<td>Formalin-killed <em>L. pneumophila</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB−</td>
<td>51.5</td>
<td>56.5</td>
<td>40.0</td>
</tr>
<tr>
<td>PB+</td>
<td>44.3</td>
<td>56.3</td>
<td>37.2</td>
</tr>
<tr>
<td>E. coli LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB−</td>
<td>55.4</td>
<td>77.7</td>
<td>60.1</td>
</tr>
<tr>
<td>PB+</td>
<td>52.3</td>
<td>54.1</td>
<td>41.8</td>
</tr>
</tbody>
</table>

*The study was similar to that in Fig. 3A, but *Legionella*-pulsed DCs were analyzed for I-A^B* (MHC class II), CD40, CD54, and CD80 expression.
pneumophila provided 100 or 90% survival against a lethal challenge with L. pneumophila, respectively (p < 0.0001, compared with naive DCs; Fig. 5A). In contrast, only 30% of mice immunized with DCs pulsed with live L. pneumophila were protected, and immunization with naive DCs provided no survival against the lethal challenge of L. pneumophila (p < 0.05, DCs pulsed with live Legionella compared with naive DCs; Fig. 5A). Although similar results were achieved with immunized BALB/c mice (data not shown), modest protection against a subsequent Legionella challenge was observed with immunized A/J mice, which are known to be genetically susceptible to Legionella infection and are useful as a mouse model of human Legionnaires’ disease (22, 23) (Fig. 5B). Immunization of A/J mice with DCs pulsed with heat- or formalin-killed L. pneumophila led to 40 or 30% survival, respectively, whereas no mice undergoing immunization of DCs pulsed with live L. pneumophila or naive DCs survived the infection of L. pneumophila (p < 0.0001 or p < 0.05, DCs pulsed with heat- or formalin-killed Legionella compared with naive DCs, respectively; p > 0.8, DCs pulsed with live Legionella compared with naive DCs; Fig. 5B).

Requirements for DC expression of MHC class I and II molecules
To assess the role of MHC Ag presentation by DCs in the induction of protective immune responses in vivo, DCs were prepared from wild-type, MHC class I-deficient, or MHC class II-deficient C57BL/6 mice for pulsing with Legionella and were used to immunize wild-type C57BL/6 mice 3 wk before intratracheal instillation of L. pneumophila (Fig. 5C). When pulsed with heat-killed L. pneumophila, MHC class II-deficient DCs provided no protection against Legionella challenge, which was not significantly different from the result with naive wild-type DCs (p > 0.6; Fig. 5C). Immunization using MHC class I-deficient DCs pulsed with heat-killed L. pneumophila conferred some protection against lethal Legionella infection compared with that using naive wild-type DCs (p < 0.05), but no mice survived until the end of the experiment on day 14 (p < 0.0001, class I-deficient DCs compared with Legionella-pulsed wild-type DCs; Fig. 5C).

Microspecific immunity
The microspecificity of protective immunity developed by Legionella-pulsed DCs was ascertained using different strains of L. pneumophila and another Gram-negative bacterium, E. coli (Fig. 5D). In these studies, A/J mice were immunized with DCs pulsed with a heat-killed clinical strain of L. pneumophila (Suzuki strain), heat-killed environmental strain of L. pneumophila, or heat-killed E. coli 3 wk before challenge with the L. pneumophila clinical strain (Suzuki strain). DC immunization with clinical and environmental strains of L. pneumophila provided comparable protection (p > 0.5), resulting in 40 and 30% survival against a lethal challenge of Legionella (p < 0.01 and p < 0.05, compared with immunization using naive DCs, respectively; Fig. 5D). In contrast,
the survival of mice immunized with E. coli-pulsed DCs against pulmonary Legionella infection was not improved ($p > 0.7$, compared with naive DCs; Fig. 5D). As a control, when mice were challenged with a lethal intratracheal infection of E. coli, no protective effect was observed with Legionella-pulsed DCs ($p > 0.8$, DCs pulsed with either strain of Legionella compared with naive DCs; data not shown).

**Surface phenotype of Legionella-pulsed macrophages**

Given that live L. pneumophila was shown to abrogate DC maturation in contrast to dead L. pneumophila, we next investigated whether a similar pattern of macrophage responses to live and dead L. pneumophila also occurs by flow cytometric analyses for their expression of I-A$^k$, MHC class II (Fig. 6A). Compared with naive macrophages, the percentage of positive cells for I-A$^k$ increased in macrophages pulsed with both heat- and formalin-killed L. pneumophila (heat-killed Legionella, 73.9 vs 49.7%; formalin-killed Legionella, 62.2 vs 49.7%; Fig. 6A), like DCs pulsed with heat- and formalin-killed L. pneumophila (Fig. 1A). When pulsed with live L. pneumophila, macrophages up-regulated MHC class II expression, as indicated by the increased proportion of I-A$^k$ cells (55.2 vs 49.7%; Fig. 6A), unlike DCs pulsed with live L. pneumophila, which strikingly down-regulated the I-A$^k$ expression (Fig. 1A).

**DC-restricted proliferative responses to live Legionella**

To further explore the responses of DCs and macrophages to L. pneumophila, we examined and characterized their proliferative phenotype, finding that DCs, but not macrophages, markedly proliferated only when pulsed with live L. pneumophila (Fig. 6B and C). In this context, DCs generated from A/J mice with GM-CSF and IL-4 were unpulsed or pulsed with heat-killed, formalin-killed, or live L. pneumophila and then cultured for 7 days without GM-CSF and IL-4 to analyze their proliferation. Only pulsing DCs with live L. pneumophila induced a significant increase in viable cell counts, and cell yields reached 269 ± 19% of the starting cell numbers at day 6 ($p < 0.0001$, DCs pulsed with live Legionella compared with all other DCs; Fig. 6B). As was seen with naive DCs, the number of DCs pulsed with heat- and formalin-killed L. pneumophila was similarly reduced starting from the initiation of the culture, but the reduction was not greater than that of naive DCs ($p > 0.2$, compared between heat- and formalin-killed Legionella; $p < 0.001$, DCs pulsed with heat- and formalin-killed Legionella compared with naive DCs; Fig. 6B). Similar results were achieved using DCs prepared from C57BL/6 mice (data not shown). In contrast, examination of macrophages after the pulse with Legionella indicated decreased numbers of viable macrophages regardless of what type of Legionella they had been pulsed with ($p > 0.05$, naive macrophages compared with all other macrophages; Fig. 6C).

The proliferation of DCs in response to live L. pneumophila was associated with significant increases in the proportion of live Legionella-pulsed DCs in the S and G2/M phase fractions ($p < 0.05$ for both S and G2/M, DCs pulsed with live Legionella compared with all other DCs; Fig. 6D). These data suggest that pulsing DCs with live L. pneumophila rescues the growth inhibition and the G0/G1 cell cycle arrest, both of which are induced by the depletion of growth cytokines. The increased proportion of live Legionella-pulsed DCs in S and G2/M phases became obscured 6 days after the initiation of the culture (data not shown).

**Discussion**

Although considerable effort has been focused on host immune mechanisms that mediate protection against infection of L. pneumophila, to date there have been no reports demonstrating the involvement of DCs. Thus, in the present study, we hypothesized that DCs have a potential role in the development of adaptive immunity to Legionella infection. Several pieces of evidence described in the present work support this hypothesis. Adoptively transferred DCs pulsed ex vivo with dead L. pneumophila, but not live L. pneumophila, elicited Legionella-specific Ig isotype responses in vivo that might have contributed to the protection against subsequent lethal challenge with L. pneumophila in a microbe-specific manner. This in vivo effect was correlated with DC phenotypic maturation that was promoted through a TLR4-dependent or TLR4-independent signaling pathway by dead L. pneumophila. Studies conducted using knockout mice suggested that the in vivo protective efficacy of Legionella-pulsed DCs was mediated by MHC class II-restricted CD4$^+$ Th cell immune responses. Taken
together with these findings, the inability of live *L. pneumophila* to trigger DC maturation and its macrophage-stimulating ability suggested that macrophages or DCs might be central to innate or adaptive immunity, respectively, against *Legionella* infection.

*Legionella* elicits immune responses mediated by CD4+ Th cells, especially Th1 cells, after infection, resulting in the generation of adaptive immunity to reinfection, as in the case of other intracellular pathogens (*e.g.*, *Listeria, Mycobacteria, Toxoplasma, Leishmania*, and *Chlamydia*) (24–29). In particular, IFN-γ, which is secreted from Th1 cells primed in primary infection, is considered to be critical for host resistance to reinfection, because IFN-γ activates mononuclear phagocytes such as monocytes and macrophages, which are primary effector cells against *L. pneumophila* (30–32). Humoral Ab responses also serve for the adaptive immunity against *Legionella* reinfection, as suggested by the following evidence: a CD4+ T cell-mediated type-specific Ab response, initially IgM followed by IgG, occurs in patients with Legionnaires’ disease, and anti-*Legionella* Abs promote the killing of *L. pneumophila* by activated phagocytes (1–4). Although this understanding of the mechanisms mediating Ag-specific host protection against *Legionella* highlights the importance of priming CD4+ T cells specific for *Legionella* protein epitopes, which APCs take on the function of CD4+ T cell priming has yet to be investigated. Hence, in this study, we evaluated the concept that DCs capture *L. pneumophila* that has been killed by innate immunity (*i.e.*, mononuclear phagocytes), mature to enhance the T cell stimulatory capacity, and present *Legionella*-derived Ags to CD4+ T cells together with costimulators (*e.g.*, costimulatory molecules and cytokines), thereby enabling CD4+ T cells to induce *Legionella*-specific adaptive immunity, as described above. Consistent with this concept, the present study demonstrated that DCs pulsed with dead *L. pneumophila*, but not DCs pulsed with live *L. pneumophila*, underwent maturation with the up-regulation of MHC class II, costimulatory (B7-1 and B7-2), adhesion (ICAM-1), and signaling molecules (CD40), and the increased production of proinflammatory cytokines (IL-12, TNF-α, IL-6, and IL-1β), and that, when adoptively transferred, dead *Legionella*-pulsed DCs induced *Legionella*-specific protective immunity in a manner dependent on MHC class II Ag presentation to CD4+ Th cells.

In regard to the type of Th cell response conferred by *Legionella*-capturing DCs, Th1 immune responses are most likely generated in vivo, because DCs pulsed with dead *L. pneumophila* released large amounts of IL-12, which is the most crucial cytokine for the differentiation of naïve T cells into IFN-γ-producing Th1 cells in vivo (33). Further evidence comes from the observation that adoptively transferred DCs pulsed with *L. pneumophila* generated elevated levels of anti-*Legionella* serum Abs in Th1-linked IgG2a and IgG3 isotypes, which well correlated with the protection of DC-immunized mice against *Legionella* challenge. However, a similar elevation was also observed in the levels of Th2-linked IgG1 serum Abs, and it is therefore not clear whether *Legionella*-capturing DCs provoke a strong predominance of Th1 immune responses in vivo. In this context, the Th2-mediated immunity induced by *Legionella*-pulsed DCs may be in part responsible for the results observed in the DC immunization-challenge experiments, because beneficial roles of Th2-related as well as Th1-related immune responses against *Legionella* infection have been demonstrated (34).

Recent studies have shown that pathogen-associated molecular patterns, components commonly found on the pathogen that are not normally found in the mammalian host, are potent activators of APCs, including macrophages and DCs, and that they are recognized by ligand-specific TLRs; for example, *E. coli* LPS signals through TLR4, whereas the cell wall components of Gram-positive bacteria and peptidoglycans from *Staphylococcus aureus* signal through TLR2 (20, 21). Until recently, the TLR engagement of components derived from *L. pneumophila* was uncertain. In the current study, analyses of DC stimulation using LPS antagonist polymyxin B and TLR4 mutant DCs revealed that TLR4 or *Legionella* LPS was involved in the DC maturation by heat- or formalin-killed *L. pneumophila*, respectively, thus suggesting that *Legionella* LPS is not recognized by TLR4. These findings support recent observations that LPS derived from *L. pneumophila* required TLR2 rather than TLR4 to stimulate mouse bone marrow granulocytes (35). Although detailed molecular mechanisms of polymyxin B-*Legionella* LPS interactions await further studies, it is conceivable that the TLR2 signaling may be, in part, responsible for the DC maturation triggered by formalin-killed *L. pneumophila*.

The present study demonstrated that, in response to live *L. pneumophila*, DCs exhibited impaired maturation in contrast to the activation seen in macrophages, suggesting that the innate immune system, such as macrophages, first attack the bacteria to kill them, and this attack is essential for DCs to establish the adaptive immunity against *Legionella* infection. Although exposure to pathogens generally gives rise to DC maturation, exceptions to this have been reported (7–10). Some pathogens have been shown to manage to avoid inducing DC maturation. For example, the parasite *Leishmania mexicana* transforms into the noninfectious amastigote form for persistent infection (36). Other pathogens are known to possess mechanisms for inhibiting DC maturation. This is illustrated by HSV-1 and vaccinia virus; HSV-1 is thought to act intracellularly, targeting a signal transduction pathway related to DC maturation, and vaccinia may secrete proteins that inhibit cytokines involved in DC maturation (37, 38). Similarly, erythrocytes infected with the malaria parasite *Plasmodium falciparum* have been shown to interfere with DC maturation (39). Although the mechanisms by which the capture of live *L. pneumophila* allows DCs to promote the cell cycle for their proliferation are elusive, the phenomenon may accompany impaired maturation of these DCs. These findings will fuel further work toward understanding the interaction between the immune system and pathogens.

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**References**


