Innate Instruction of CD4+ T Cell Immunity in Respiratory Bacterial Infection

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The innate immune system recognizes invading microbes via conserved pattern recognition receptors and uses inflammatory signals to concert adaptive defense mechanisms. However, microbial and host parameters involved in CD4 T cell priming and direction of Th1, Th2, and Th17 differentiation in the context of infections with complex pathogens in vivo are incompletely understood. In this study, we used *Legionella pneumophila*, which triggers membrane-bound and cytosolic pattern recognition receptors, to study the innate instruction of adaptive immunity. Upon airway infection, T cells were primed exclusively in the lung-draining lymph nodes and differentiated into Th1/Th17 effector cells upon arrival in the lung. Although engagement of membrane-bound pattern recognition receptors was sufficient for initial T cell activation and proliferation, cytosolic pattern recognition was required for effector T cell differentiation. In the absence of cytoplasmic pattern recognition, MyD88 was key for T cell priming, whereas, in its presence, MyD88-mediated signals were crucial for Th17 differentiation. Specifically, cytosolic sensing of *Legionella*-derived flagellin, inflammasome activation, and IL-1 signaling contributed to Th17 development. In the absence of TLR signaling, a simultaneous Th1/Th2 response developed that was independent of the inflammasome–IL-1 axis. Collectively, these data illustrate the important role for various pattern recognition receptors triggered by complex pathogens and how they each instruct specific differentiation programs in responding CD4 T cells. *The Journal of Immunology, 2012, 189: 616–628.*

The vertebrate immune system has evolved potent mechanisms that efficiently protect us from recurrent infections. The innate immune system, with a limited repertoire of germline-encoded pattern recognition receptors (PRRs), serves as a first line of defense, which gets activated very quickly upon infection and recognizes evolutionarily conserved patterns of pathogenic invaders. If innate immune mechanisms are not sufficient to control an infection, the adaptive immune system provides a second line of defense. However, to prevent autoimmune diseases, there is a need for tight control of adaptive immune cells, provided, among others, by instructive signals of innate immune cells, which are crucial for initial activation, as well as for adjustment of adaptive defense mechanisms to the respective pathogen.

CD4 T cells have a prominent regulatory role among the cellular players of the immune system. To fulfill their specific requirements, CD4 T cells differentiate into different T cell lineages, which are highly specialized toward different pathogens. Initial recognition of these invaders is mediated by the family of signaling PRRs (1), which are positioned at strategically important subcellular locations within innate immune cells. Among them, membrane-anchored TLRs are located at cell surfaces or in endosomal compartments, whereas Nod-like receptors (NLRs) survey the cytoplasm. Upon activation, these receptors trigger the expression of genes that provide the required information for priming of CD4 T cells: expression of costimulatory molecules (signal 2) that enhance T cell responses and production of cytokines (signal 3) that trigger the transcription of master transcription factors in CD4 T cells and guide them into the Th1, Th2, Th17 or regulatory T cell lineage. Understanding this process of innate instruction of adaptive immune responses is of fundamental importance for the interpretation of the pathophysiology of diseases like allergy and asthma, as well as for the development of appropriate vaccination strategies. However, the signals and mechanisms that guide CD4 T cell lineage differentiation in vivo are not completely understood, in particular in the context of complex pathogens.

Most approaches attempting to characterize innate receptors and their role in influencing adaptive immune responses have used purified pathogen-associated molecular patterns (PAMPs). Although, using a limited and controlled stimulus for studying immune responses has certain advantages, it can never simulate the dynamic and highly complex immune response during a natural infection. Only living pathogens provide natural attributes termed “patterns of pathogenesis,” such as virulence, growth, cytosolic access, or even interruption of normal host functions (2) and, therefore, remain invaluable tools to trigger and understand complex immune responses. The Gram-negative bacterium *Legionella pneumophila*, the causative agent of Legionnaires’ disease (3, 4), possesses all of these characteristics and was shown to trigger a number of PRRs. Inhalation of *L. pneumophila*-contaminated aerosols results in bacterial uptake and intracellular replication within alveolar macrophages. The ability of *L. pneumophila* to escape lysosomal degradation after phagocytic uptake presents a particular challenge to the host immune system. *L. pneumophila* modulates the host cell via cytosolic injection of virulence factors through its Icm/Dot type IV secretion system (T4SS) (5), which enables the bacterium to replicate in a specialized nondegradative
vacuole. It triggers transmembrane TLRs (7, 8), as well as cytosolic NLRs (9, 10), and induces a potent inflammatory response, leading to the subsequent initiation of a robust adaptive immune response in the host. Abs of the IgG and IgA isotype were shown to protect from *L. pneumophila* infection, and CD4 T cells were crucial for Ab isotype switch (11, 12). In an *AJ* mouse model, CD4 T cells were demonstrated to mediate protection in primary infection (13). Although *L. pneumophila* is a bacterium that resides in a vacuole that does not fuse with lysosomes, induction of *L. pneumophila*-specific CD4 T cells occurs in vivo (14). However, the exact site and kinetics of T cell priming and differentiation in vivo, as well as the instructive innate components that contribute to CD4 T cell differentiation, have not been investigated.

We used *L. pneumophila* as a model organism in an airway-infection system to study the innate components involved in pathogen recognition and induction of signals that lead to the instruction of CD4 T cell immunity in vivo. Because no *L. pneumophila*-specific T cell epitopes have been identified, we generated an *L. pneumophila*-compatible vector encoding for a GFP-OVA fusion protein as an *L. pneumophila*-inherent neoantigen with known TCR specificities. Transformation of this vector into different *L. pneumophila* knockout strains enabled us to analyze the influence of secreted *L. pneumophila*-derived PAMPs and virulence factors on innate recognition by APCs, as well as the subsequent activation of OVA-specific TCR-transgenic CD4 T cells (OT-II cells) in vitro and in vivo. Using the intranasal (i.n.) route as the natural way of infection, we show that *L. pneumophila*-specific CD4 T cells get primed and proliferate in the mediastinal lymph node (MLN) and differentiate into Th1/Th17 effector cells upon arrival in the lung. On the pathogen side, this process strictly depends on a functional Icm/Dot T4SS for Th1/Th17 differentiation and on flagellin for Th17 differentiation. On the host side, TLR signaling was important for the generation of Th1/Th17 cells, because infection of MyD88−/− mice triggered a mixed Th1/Th2 response and severely hampered the development of Th17 cells. Interestingly, in the absence of cytosolic pattern recognition, MyD88 was key for CD4 T cell priming in the MLN. Moreover, Th17 development was dependent on inflammasome activation, because caspase-1−/−, IL-1R−/−, and IAPF−/− mice showed decreased differentiation into *L. pneumophila*-specific Th17 cells. Thus, these data provide insight into the role of various PRRs and adapter molecules involved in the generation and transmission of signals whose integration is crucial for the formation of an appropriate Th1/Th17 response in the *L. pneumophila* infection model.

Materials and Methods

**Mice, bacteria, and infections**

C57BL/6, Myd88−/− (15), IL-1R−/− (16), IL-18−/− (17), caspase-1−/− (18), and IAPF−/− mice were bred at the Swiss Federal Institute of Technology Zürich or purchased from Janvier Elevage and used at 6–12 wk of age (age- and sex-matched within experiments). All mice were backcrossed on C57BL/6. All animal experiments were performed in accordance with institutional policies and were reviewed by the cantonal veterinary office. The *L. pneumophila* strains used in this study were JR32 (wild-type [WT] [Philadelphica-1]) (19), JR32-GFP (20), JR32-GS3011 (ΔT, icmT deletion mutant lacking a functional Icm/Dot T4SS) (21), ΔT-GFP (20), and Corby (WT or ΔFlaA mutant) (8). *L. pneumophila* was grown for 3 d on charcoal yeast extract agar plates. Liquid cultures were inoculated in ACES yeast extract medium at an OD600 of 0.1 and grown for 21 h at 37°C. Chloramphenicol (5 mg/ml) was added for selection. Mice were anesthetized with ketamine (1.9 mg) and xylazine (0.1 mg) and infected i.n. with 5×10^6 CFU *L. pneumophila* (grown on plates) in 20 μl prewarmed PBS that was applied directly onto the nostril using a Gilson pipette. After sublethal anesthesia with xylazine (0.38 mg), ketamine (1.9 mg), and acepromazine (57 μg), mice were perfused with PBS. CFU counts were determined by plating diluted of homogenized lung tissue onto charcoal yeast extract agar plates.

**Cloning of GFP-OVA construct**

DNA encoding GFP was amplified from the plasmid pNT28 (20) with the primers pNT28-GFP-Munl-3′ (5′-CACAATCCCTATGCCGTC-3′) and pNT28-GFP-BamHi-Nhel-3′ (5′-AAATGCTAGCTAATGAGGAGAT-3′) for insertion of linker sequences between BamHI and Nhel and cloned into the multiple cloning site of the packaging vector pMD-Teasy (Promega). OVA was amplified from the plasmid pDP087, which was kindly provided by Daniel Pinschewer (University of Geneva, Geneva, Switzerland), with the primers Nhel-OVA-5′ (5′-TAAGTTATCGTGATAGTTGAGAT-3′) and OVA-Stop-MulI-3′ (5′-GGTCACCGGTCAAGGAGAACCATGCT-3′) and cloned via Nhel and MulI into the pMD-Teasy vector. A 6x Gly linker was inserted between BamHI and Nhel using the DNA-oligonucleotides 5′-BamHi-GSPAATTTTCTGAGGAT-3′ and 5′-BamHI-GSpNheI-3′ (5′-GCTAGACCAACCACCCACCAAGGGAATTCGCTAGATT-3′). DNA encoding GFP-6×Gly-OVA was amplified from pMD-Teasy using the primers 5′-EcoRI-TTRBS (5′-GGAAGGTGAATTCGCTAGATT-3′) and 3′-OVA-HindIII (5′-TTAGCTAGCTTAAGAGAGAT-3′) and cloned into the L. pneumophila ΔT strain (8) were transformed with pGT1, resulting in GFP-OVA-expressing strains.

**Abs, intracellular cytokine staining, and flow cytometry**

All Abs for FACS analysis were from BioLegend (Luzern, Switzerland) or BD Biosciences (Allschwil, Switzerland). Lungs were digested for 30 min at 37°C with Collagenase I (Life Technologies, Invitrogen, Basel, Switzerland) and DNase I (Roche Diagnostics, Rotkreuz, Switzerland), and a single-cell suspension was prepared by forcing the organ through a 70-μm strainer. Cells from the lung were stained by standard procedures using fluorescence-labeled Abs. Thioglycolate-induced peritoneal macrophages were prepared by i.p. injection of thioglycolate (4% in PBS) and were used as APCs for restimulation of T cells. For intracellular cytokine staining (ICC), 3×10^5 of endogenous T cells, APCs were cultured with *L. pneumophila* lysate for 5 h before the addition of lung or MLN lymphocytes. For ICCS of OT-II Ly5.1 cells from lung or MLN, APCs were loaded with OVA peptide (aa 323–339, 106 M). Cultures were kept for 5 h in the presence of brefeldin A and monensin prior to ICCS. Cells were surface stained for 30 min at 4°C, fixed, and permeabilized in 500 μl 2× FACS Lyse (BD Biosciences) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO) for 10 min at room temperature. After washing, ICCS was performed for 30 min at room temperature. Cells were washed again before resuspension in PBS containing 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Intracellular T-bet and Gata-3 staining was performed using the Fop3x staining kit, according to the manufacturer’s protocol (ebioscience, San Diego, CA). Data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR).

**Adaptive T cell transfers**

OT-II Ly5.1 cells were isolated from the spleen of OT-II Ly5.1 TCR-transgenic mice, purified to ≥90% with CD4 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and labeled with 0.25 μM CFSE (Invitrogen, Karlsruhe, Germany) before injection into congenic recipients. For analysis of proliferation on day 3 postinfection (p.i.), 5×10^6 OT-II Ly5.1 cells were transferred; for analysis on day 6 p.i., 10^6 OT-II Ly5.1 cells were transferred. Adoptive transfers were performed 1 d before infection.

**Statistical analysis**

The two-tailed unpaired *t* test was applied for statistical analysis, using Prism GraphPad software (La Jolla, CA).

**Results**

Legionella-specific CD4 T cells exhibit a Th1/Th17 effector phenotype

*L. pneumophila*-specific adaptive immunity has been implicated in the control of pulmonary infection (11, 13, 22–27); however, there is limited information on the differentiation and effector functions...
of \textit{L. pneumophila}-specific CD4 T cells in vivo. In particular, the influence of \textit{L. pneumophila}-encoded virulence factors, including the \textit{L. pneumophila}-encoded T4SS, on in vivo CD4 T cell priming, proliferation, lineage differentiation, and induction of effector functions is still unknown. Therefore, we analyzed endogenous \textit{L. pneumophila}-specific CD4 T cell responses after i.n. infection with either WT \textit{L. pneumophila} or a \textit{ΔT} mutant strain, which does not express a functional T4SS. The \textit{ΔT} mutant \textit{L. pneumophila} is targeted to the phagolysosomal pathway for degradation (6, 28, 29) and is a poor inducer of inflammation in vivo compared with WT \textit{L. pneumophila} (8, 30). Thus, by comparing infections with WT and \textit{ΔT} mutant \textit{L. pneumophila}, CD4 T cells are either primed in a highly inflammatory environment and in the presence of cytotoxic PRR triggering or in the absence of both. To normalize for bacterial load (WT \textit{L. pneumophila} are able to replicate intracellularly, whereas \textit{ΔT} mutant \textit{L. pneumophila} are degraded), we used thymidine auxotroph WT and \textit{ΔT} mutant \textit{L. pneumophila}, neither of which can replicate intracellularly. Six days p.i., lymphocytes from the lungs were isolated and restimulated with an \textit{L. pneumophila} lysate in the presence of APCs, and cytokines produced by CD4 T cells were measured by ICCS. Although infection with WT \textit{L. pneumophila} resulted in a Th1/Th17 response with few IFN-γ and IL-17 double producers, CD4 T cells from mice that were infected with the \textit{ΔT} mutant strain completely failed to produce effector cytokines (Fig. 1A–C). These results indicate that the inflammatory environment generated in vivo upon \textit{L. pneumophila} infection and secretion of effector molecules and PAMPs via the T4SS have a strong influence on the induction of CD4 T cell effector functions in the lung. Alternatively, the lack of cytokine-producing CD4 T cells upon \textit{ΔT} mutant \textit{L. pneumophila} infection is also compatible with absent CD4 T cell priming. To distinguish between the absence of \textit{L. pneumophila}-specific CD4 T cell priming and effector cell differentiation, \textit{L. pneumophila}-specific CD4 T cells have to be tracked physically and not only functionally.

Recombinant expression of OVA as a neoantigen in Legionella activates OVA-specific CD4 T cells in vitro

To track \textit{L. pneumophila}-specific CD4 T cells on a physical level, we generated an \textit{L. pneumophila}-compatible vector encoding for an OVA-GFP fusion protein as a neoantigen that was introduced into WT \textit{L. pneumophila}, an \textit{L. pneumophila} \textit{ΔT} mutant strain, and an \textit{L. pneumophila} \textit{Δ FlaA} mutant strain. WT and knockout strains showed equal GFP signals, indicating equal expression of OVA (Supplemental Fig. 1). Infection of APCs in vitro with GFP-OVA \textit{L. pneumophila} and coinoculation of these cells with CFSE-labeled OVA-specific TCR-transgenic CD4 T cells (OT-II) resulted in their proliferation and differentiation into IFN-γ- and IL-17–producing cells (Fig. 2), reminiscent of the previous in vivo observations with endogenous CD4 T cells (Fig. 1). In contrast, APCs infected with GFP-expressing control \textit{L. pneumophila} did not support effector OT-II cell differentiation.

Because GFP-OVA is encoded on a plasmid, it is conceivable that the plasmid might be rapidly lost in vivo under nonselecting conditions. To assess the longevity of plasmid retention in vivo, mice were infected i.n. with GFP-OVA \textit{L. pneumophila}. Three days p.i., lungs were isolated, homogenized, and plated in the presence or absence of antibiotic selection. Approximately 80% of the isolated bacteria still expressed GFP (Supplemental Fig. 2), demonstrating that the OVA-expressing plasmid is expressed in most bacteria until day 3, which is long enough to allow for CD4 T cell priming.

\textbf{OVA-expressing Legionella prime OVA-specific CD4 T cells in vivo}

Next, we analyzed whether i.n. infection with GFP-OVA–expressing \textit{L. pneumophila} primed OT-II cells in vivo. We established an adoptive T cell transfer system, using GFP-OVA \textit{L. pneumophila} for infection via the i.n. route and OVA-specific CD4 T cells from OT-II mice carrying Ly5.1 as a congenic marker. To determine the anatomical site of OT-II cell priming, we transferred CFSE-labeled OT-II cells into recipient mice and infected them i.n. 1 d posttransfer with GFP-OVA \textit{L. pneumophila}. Isolation of lymphocytes from MLNs, lung, and spleen at different time points p.i. revealed that OT-II cells were primed and started to proliferate in the MLNs 3 d p.i. At this time point, we did not observe T cell proliferation in the lung (Fig. 3A, 3C) or in the spleen (data not shown). By day 6 p.i., heavily proliferated OT-II cells, which had outdiluted CFSE, were prominent in the lung, confirming that CD4 T cell priming and initial proliferation are

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![FIGURE 1. Legionella-specific CD4 T cells exhibit a Th1/Th17 effector phenotype. C57BL/6 mice were infected with 10⁷ CFU of thymidine auxotroph WT \textit{L. pneumophila} or \textit{ΔT} \textit{L. pneumophila}. On day 6 p.i., lung cells were isolated and restimulated with \textit{L. pneumophila} lysate prior to ICCS and FACS analysis. (A) Representative FACS plots of IFN-γ, IL-17, and IL-4 production in CD4 T cells. Plots are gated on CD4 T cells. Numbers indicate the percentage of cytokine-producing CD4 T cells. Percentages (B) and absolute numbers (C) of cytokine-producing CD4 T cells are shown. Each symbol represents one mouse. Data shown are representative of two independent experiments. ***p < 0.001.](image-url)
restricted to the MLNs upon *L. pneumophila* infection (Fig. 3B, 3D). Infection of recipient mice with GFP *L. pneumophila* did not result in priming and proliferation of OT-II cells (Fig. 3A–D).

Next, we asked whether the restricted priming of OT-II cells in the MLN is associated with the presence of live bacteria in the MLN. Therefore, we quantified CFU from lung and MLNs 2 d after *L. pneumophila* infection. Our results show that the infection is limited to the lung tissue and the bronchoalveolar space, because we were unable to recover any CFU from the MLN (Fig. 3E). Because T cell priming apparently occurs in the MLN, *L. pneu-

**FIGURE 2.** OVA-specific CD4 T cells recognize rGFP-OVA *Legionella*. OT-II Ly5.1 cells were labeled with CFSE and cultured with splenic DCs, which were previously infected with *L. pneumophila*-expressing GFP-OVA or GFP at a multiplicity of infection of 10. Cells were cultured for 5 d, and cells were peptide restimulated prior to ICCS and FACS analysis. Data shown are gated on OT-II Ly5.1 cells and are representative of two independent experiments.

**FIGURE 3.** OVA-expressing *Legionella* prime OVA-specific CD4 T cells in vivo. A total of 5 × 10^6 (A, C) or 10^6 (B, D) OT-II Ly5.1 cells were labeled with CFSE and adoptively transferred into congenic recipients. One day posttransfer, mice were infected i.n. with 5 × 10^6 *L. pneumophila* GFP-OVA. MLN and lungs of recipient mice were analyzed for the presence of transferred OT-II Ly5.1 cells in combination with CD44 staining and CFSE dilution analysis at day 3 (A, C) or day 6 (B, D) p.i. Percentage of proliferated CFSElow OT-II Ly5.1 cell in MLN and lung at day 3 (C) and day 6 p.i. (D). Data shown are gated on OT-II Ly5.1 cells and are representative of three independent experiments. (E) CFU in MLNs and lungs at day 2 p.i. Data shown are representative of two independent experiments. (F and G) MLN and lungs of recipient mice were isolated, cells were peptide restimulated in the presence of APCs, and cytokine secretion potential was measured. Data shown are representative of two independent experiments. (F) Representative FACS plots of OT-II cells from MLN and lung at day 6 p.i. (G) Percentages of IFN-γ+ and IL-17+ OT-II cells from MLN and lung at the indicated time points p.i. ***p < 0.001.
mophila Ags have to be transported to the MLN from the lung tissue, either by migratory APCs or, less likely, as soluble lymph node-drained Ags. Interestingly, OT-II cells did not produce cytokines in the MLN (at 3 or 6 d p.i.), but started to produce cytokines as soon as they were present in the lung tissue at day 6 p.i. (Fig. 3F, 3G), indicating an important role for the inflammatory lung environment in promoting effector cell functions of OT-II cells or alternatively indicating that only highly proliferated cells, which we do not find in the MLNs (Fig. 3A, 3B), exhibit cytokine production potential.

Initial CD4 T cell activation is supported by T4SS-deficient Legionella

Based on our initial observation that cytokine-producing endogenous L. pneumophila-specific CD4 T cells are not detected upon ΔT mutant L. pneumophila infection (Fig. 1), we addressed the question whether L. pneumophila-specific CD4 T cells are primed in this setting. CFSE-labeled OT-II cells were adoptively transferred into recipient mice; 1 d posttransfer, recipient mice were infected i.n. with GFP-OVA L. pneumophila or GFP-OVA ΔT L. pneumophila. On day 3 p.i., lymphocytes from the MLN were isolated, and CFSE dilution was measured. OT-II cells proliferated vigorously in the MLN following infection with WT L. pneumophila or ΔT L. pneumophila (Fig. 4A). Interestingly, in the majority of our experiments we observed even stronger proliferation of OT-II cells on day 3 p.i. with ΔT L. pneumophila, suggesting that L. pneumophila-derived Ags might be more efficiently processed and presented on MHC class II molecules from lysosomal compartments compared with Ags from the L. pneumophila-containing vacuole (Fig. 4A). These findings show that cytotoxic recognition of L. pneumophila-derived PAMPs and effector molecules and overt inflammation are not essential for early activation and subsequent proliferation of L. pneumophila-specific CD4 T cells in the MLN. However, when we analyzed OT-II cell numbers and proliferation at day 6 p.i., it became apparent that both were substantially reduced in GFP-OVA ΔT L. pneumophila-infected mice compared with WT L. pneumophila-infected mice (Fig. 4B, 4C). Although infection of OT-II recipient mice with GFP-OVA L. pneumophila WT resulted in ongoing proliferation of OT-II cells in the MLN (Fig. 4B) and arrival of proliferated CFSElow OT-II cells in the lung at day 6 p.i. (Fig. 4C), the extent of proliferation of OT-II cells in the MLN was smaller and total numbers of OT-II cells were significantly lower 6 d after GFP-OVA ΔT L. pneumophila infection (Fig. 4B). Reduced numbers of OT-II cells after GFP-OVA ΔT L. pneumophila infection in the MLN were not based on faster migration of proliferated OT-II cells to the lung, because they were also severely reduced in the lung after GFP-OVA ΔT L. pneumophila infection (Fig. 4C). These results show that cytotoxic pattern recognition of L. pneumophila-derived PAMPs and/or the presence of an inflammatory environment is crucial for sustained CD4 T cell proliferation, survival, and/or migration from the MLN to the lung.

T4SS-deficient Legionella do not support effector cell differentiation

Next, we analyzed effector cell differentiation upon WT or ΔT mutant L. pneumophila infection. Consistent with the endogenous L. pneumophila-specific CD4 T cell response (Fig. 1A, 1B), OT-II cells also differentiated into Th1/Th17 effector cells in recipient mice upon infection with GFP-OVA L. pneumophila. In contrast, although infection of recipient mice with GFP-OVA ΔT L. pneumophila induced initial (albeit reduced) proliferation of OT-II cells, production of IFN-γ and IL-17 by OT-II cells in the lung was completely absent (Fig. 5). These results demonstrate that cytosolic pattern recognition of L. pneumophila-derived PAMPs and/or the presence of an inflammatory environment is decisive for differentiation of L. pneumophila-specific CD4 T cells into effector cells upon arrival in the airways.

Legionella-derived flagellin promotes Th17 responses

Previous studies indicated that effector molecules, as well as PAMPs like flagellin, can access the cytosol of host cells via L. pneumophila T4SS. In addition, TLR5-mediated recognition of flagellin was reported to be involved in CD4 T cell lineage differentiation (31). Based on our finding that a functional T4SS plays a crucial role in the differentiation of L. pneumophila-specific CD4 T cells, we addressed the potential role for flagellin in this differentiation process. To this end, OT-II cells were adoptively transferred into recipient mice that were infected i.n. with GFP-OVA L. pneumophila or GFP-OVA ΔFlaA L. pneumophila, which do not express flagellin, 1 d posttransfer. At day 6 p.i., lymphocytes were isolated from the lung, and CD4 T cells were analyzed for effector functions. Although WT L. pneumophila induced a Th1/Th17 response, infection with ΔFlaA L. pneumophila resulted in impaired IL-17 responses in OT-II cells upon i.n.
FIGURE 5. ΔT Legionella do not support effector cell differentiation. A total of 10⁶ OT-II Ly5.1 cells was labeled with CFSE and adoptively transferred into congenic recipients. One day posttransfer, mice were infected i.n. with 10⁵ thymidine auxotroph L. pneumophila GFP-OVA, L. pneumophila ΔT GFP-OVA, or L. pneumophila GFP. MLN and lungs of recipient mice were analyzed for the presence of transferred OT-II Ly5.1 cells, CFSE dilution, and cytokine production at day 6 p.i. (A and C) Representative FACS plots showing proliferation and cytokine production of OT-II cells in the lung on day 6. Plots are gated on OT-II cells. (B and D) Absolute numbers and percentages of cytokine-producing OT-II cells in the lung at day 6. Each symbol represents one mouse. Data shown are representative of two independent experiments. *p < 0.05.

infection (Fig. 6A, 6B), but no difference in IFN-γ responses (Fig. 6C, 6D), suggesting that innate recognition of L. pneumophila-derived flagellin plays a decisive role in driving IL-17 production potential within L. pneumophila-specific CD4 T cells. Of note, bacterial titers were higher in ∆FlaA L. pneumophila-infected mice, particularly at day 3 p.i. (Fig. 6E), which might explain the slightly higher frequencies of IFN-γ-producing OT-II cells due to increased Ag load. Despite this increase in Ag load, IL-17-producing cells were markedly reduced in association with ∆FlaA L. pneumophila infection.

A mixed Th1/Th2 response is induced in the absence of MyD88

While our previous experiments examined the role of L. pneumophila-derived PAMPs in the induction of L. pneumophila-specific CD4 T cell responses, we now focused on the contribution of host PRRs in directing adaptive immune responses. MyD88 is a key adapter molecule involved in signaling of most TLRs and mediating proinflammatory signaling via IL-1R and IL-18R. Because signaling via these pathways can influence the size and type of the adaptive immune responses, we investigated whether the absence of MyD88 affects adaptive immune responses during L. pneumophila infection. Interestingly, endogenous CD4 T cells from lungs of MyD88−/− mice secreted IL-4 and showed severely impaired IL-17 production after i.n. L. pneumophila infection (Fig. 7A–C). Moreover, MyD88−/− mice supported the differentiation of IL-4– and IFN-γ–producing CD4 T cells at the same time, with even some IL-4/IFN-γ double-producing cells (Fig. 7D, 7E), supporting the recently emerging evidence for plasticity of CD4 T cell subsets (32) also during L. pneumophila infection.

To confirm our findings on the induction of a simultaneous Th1/Th2 response in MyD88−/− mice on a monoclonal level, we infected MyD88−/− mice after OT-II transfer with GFP-OVA L. pneumophila. At day 6 p.i., OT-II cells from the airways had developed into Th1 and Th2 cells (Fig. 8A), with some IFN-γ/IL-4 double producers (Fig. 8B). Intracellular staining for the Th1 and Th2 master transcription factors T-bet and Gata-3 confirmed these results and revealed coexpression of T-bet and Gata-3 in OT-II cells in MyD88−/− mice. Interestingly, OT-II cells that had already arrived in the lung showed higher expression of these two master transcription factors compared with OT-II cells that were isolated from the MLN (Fig. 8C–E). This finding correlates with the fact that OT-II cells produce cytokines only in the lung, and not in the MLN, after L. pneumophila infection.

Previous studies showed that the amount of Ag and PAMPs can influence the induction of Th1 or Th2 responses (33, 34). Due to severe defects in the innate immune response in MyD88−/− mice, the pathogen load in lungs of infected MyD88−/− mice is highly increased compared with C57BL/6 mice (Fig. 7F). To analyze a potential role for the increased pathogen load in Th2 induction in MyD88−/− mice, we adoptively transferred OT-II cells into MyD88−/− mice and infected the recipients with proliferating GFP-OVA L. pneumophila or with a thymidine auxotroph strain of GFP-OVA L. pneumophila, thereby normalizing for L. pneumophila loads in WT and MyD88−/− animals. Independent of the pathogen load, OT-II cells still developed into Th1 and Th2 cells in MyD88−/− mice, although infection with thymidine auxotroph L. pneumophila induced an overall reduction in the size of effector cell pool (Fig. 8F, 8G). This result suggests that proliferation of L. pneumophila and, hence, the high amount of Ag and PAMPs, promotes CD4 T cell responses in general, but is not critical for the induction of Th2 responses in MyD88−/− mice.

Next, we investigated whether Th2 induction in MyD88−/− mice is based on the absence of signals mediated via IL-1R or whether missing TLR signals are responsible. Therefore, we analyzed induction of Th2 responses using our adoptive-transfer system in...
caspase-1−/− mice, which lack the production of mature IL-1 and IL-18 proteins. In addition, we tested for Th2 induction in IL-1R2−/− mice. We did not observe induction of IL-4–producing OT-II cells in any of these mice upon *L. pneumophila* infection, indicating that a reduced or missing Th1-inducing signal, mediated via TLRs, most likely allows for Th2 induction (Supplemental Fig. 3A).

Previous studies showed that *L. pneumophila*-derived flagellin can induce Th2 responses (31). Because Th2 induction is MyD88 independent in *L. pneumophila* infection, we were looking for an active MyD88-independent flagellin-mediated Th2-inducing signal that might become dominant in the absence of Th1-favoring TLR signals. However, infection of MyD88−/− mice with ΔFlaA *L. pneumophila* still resulted in increased Gata-3 expression, comparable to infection of MyD88−/− mice with WT *L. pneumophila*, indicating that MyD88-independent recognition of flagellin is not responsible for active Th2 induction (Supplemental Fig. 3B, 3C).

MyD88 is key for CD4 T cell priming in vivo in the absence of cytosolic pattern recognition

Our previous results showed that, in the absence of cytosolic pattern recognition (i.e., p.i. of mice with ΔT *L. pneumophila*), *L. pneumophila*-specific CD4 T cells do not differentiate into effector cells in the lung. However, CD4 T cells still get primed in the MLN and subsequently migrate to the lung, albeit at reduced numbers. In addition, our experiments revealed a crucial role for MyD88-mediated signals in the induction of a Th17 response, because the absence of MyD88 resulted in a mixed Th1/Th2 response in vivo. Based on these findings, we assessed whether there is still CD4 T cell priming in the absence of MyD88 and cytosolic pattern recognition in vivo. Infection of MyD88−/− mice with ΔT *L. pneumophila* GFP-OVA resulted in a virtual absence of OT-II cell priming in the MLN 3 d p.i. compared with ΔT *L. pneumophila*-infected C57BL/6 mice (Fig. 9). These results clearly demonstrate a crucial role for MyD88 in the transmission of signals from membrane-bound PRRs, most likely TLRs, which are essential for CD4 T cell priming.

**IL-1 and the IPAF inflammasome promote Th17 development in vivo**

Recent studies on the innate immune response to *L. pneumophila* demonstrated that inflammasome activation via recognition of *L. pneumophila*-derived flagellin plays a critical role in the secretion of IL-1β (35–37). It is well established that IL-1 has a strong influence on CD4 T cell differentiation, mainly toward the Th17 lineage (38–41). Therefore, we investigated whether IL-1 induction, mediated by activation of the inflammasome and caspase-1,
contributes to the development of Th17 responses in *L. pneumophila* infection in vivo. To this end, we adoptively transferred OT-II cells into caspase-1−/−, IL-1R−/−, and IL-18−/− recipient mice and infected them i.n. with GFP-OVA *L. pneumophila*. Isolation of lung lymphocytes and analysis for Th17 cells on day 6 p.i. revealed that, similarly to MyD88−/− recipient mice, IL-17+ cells were reduced in the lungs of caspase-1−/− and IL-1R−/− mice compared with C57BL/6 mice (Fig. 10A). Interestingly, IL-18−/− mice showed markedly increased amounts of Th17 cells, perhaps due to the missing competition between pro–IL-1 and pro–IL-18 for processing by caspase-1. These results suggest that inflammasome activation and processing of IL-1 upon *L. pneumophila* infection contribute to the development of Th17 cells. Furthermore, IL-1 signaling was not directly acting on the differentiating CD4 T cells, because Th17 induction was also impaired when IL-1R−/− recipient OT-II cells were transferred into IL-1R−/− hosts.

Previous in vitro studies demonstrated that *L. pneumophila*-derived flagellin activates the IPAF inflammasome (35–37). Based on our findings that flagellin promotes Th17 responses and Th17 differentiation depends on IL-1 signaling, we next asked whether...
the IPAF inflammasome contributes to Th17 development in vivo. To this end, we infected IPAF−/− mice, which had received OT-II cells, with GFP-OVA L. pneumophila i.n. and analyzed the frequency of Th17 cells in the lung. We observed a trend toward a reduced amount of Th17 cells in IPAF−/− mice compared with C57BL/6 mice (Fig. 10B), suggesting that activation of the IPAF inflammasome via L. pneumophila-derived flagellin contributes to Th17 development of L. pneumophila-specific CD4 T cells.

Discussion
In this article, we addressed the question of how pattern recognition by innate receptors contributes to the shaping of CD4 T cell
responses in the context of experimental airway infection with *L. pneumophila*. Although previous studies reported a Th1 response in the context of *L. pneumophila* infection (42, 43), we show in vitro and in vivo that *L. pneumophila*-specific CD4 T cells also differentiate into Th17 cells. Most previous in vivo studies of adaptive immune responses used the A/J mouse (11, 13, 14), which harbors a natural mutation in the Naip5 gene locus, resulting in reduced function of the NLR Naip5 protein (9, 44). Naip5 was shown to be involved, together with the NLR IPAF, in the cytosolic recognition of *L. pneumophila*-derived flagellin, resulting in secretion of IL-1β by macrophages (35). Because IL-1 is well known to promote Th17 development (38–41), either directly or indirectly (e.g., via activation of γδ T cells) (45), an impaired recognition of *L. pneumophila*-derived flagellin in A/J mice could account for decreased IL-1 secretion and, subsequently, impaired Th17 differentiation. Alternatively, the different genetic background compared with C57BL/6 mice could be responsible for the absence of Th17 cells. Indeed, our experiments in A/J mice showed reduced frequencies of *L. pneumophila*-specific Th17 cells compared with C57BL/6 mice (G. Trunk and A. Oxenius, unpublished observations). Consistent with a role for IL-1 in the in vivo differentiation of Th17 cells during *L. pneumophila* infection, our results show compromised Th17 differentiation in the absence of MyD88, caspase-1, IL-1, and, to some extent, IPAF, indicating an important role for inflammasome activation and the subsequent secretion of IL-1 in the promotion of

**FIGURE 9.** MyD88 is key for priming of *Legionella*-specific CD4 T cells in vivo after ΔT *Legionella* infection. A total of $3 \times 10^6$ OT-II L5.1 cells was labeled with CFSE and adoptively transferred into congenic C57BL/6 or MyD88−/− recipients. One day posttransfer, mice were infected i.n. with $5 \times 10^6$ *L. pneumophila* GFP-OVA or *L. pneumophila* ΔT GFP-OVA. MLNs of recipient mice were analyzed for the presence of transferred OT-II cells, CFSE dilution, and T-bet upregulation at day 3 p.i. (A) Representative FACS plots showing CFSE dilution and T-bet expression in *L. pneumophila*-specific CD4 T cells. (B) Percentages (left panel) and absolute numbers (right panel) of proliferated CFSElow OT-II cells. Data shown are representative of two independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 10.** IL-1 and the IPAF inflammasome promote Th17 development in vivo. A total of $10^6$ OT-II L5.1 cells was labeled with CFSE and adoptively transferred into WT or knockout congenic recipients. One day posttransfer, mice were infected i.n. with $5 \times 10^6$ *L. pneumophila* GFP-OVA. Lungs of recipient mice were analyzed for the presence of transferred OT-II L5.1 cells and their cytokine-secretion potential after peptide restimulation at day 6 p.i. (A and B) Absolute numbers and percentages of IL-17–producing OT-II cells in the lung at day 6 p.i. Each symbol represents one mouse. Data shown are representative of two independent experiments. **p < 0.01, ***p < 0.001.
Th17 responses. L. pneumophila-derived flagellin was shown to activate cell surface TLR5 (46); however, it can also access the cytosol via L. pneumophila T4SS, where it activates the NLRs IPAF and Naip5 (35–37, 47). Because both cytosolic PRRs were shown to activate caspase-1 and, thereby, induce the secretion of IL-1β (37, 48), this likely explains the fact that Th17 responses were less impaired in the sole absence of IPAF compared with the absence of MyD88, caspase-1, or IL-1R.

Our analysis of the endogenous CD4 T cell response revealed no induction of cytokine-producing L. pneumophila-specific CD4 T cells upon infection with L. pneumophila devoid of a functional T4SS, correlating with impaired induction of an inflammatory response (30). Whether the absence of effector functions in L. pneumophila-specific CD4 T cells was based on a defect in priming or effector cell differentiation was addressed using an adoptive T cell transfer system with GFP-OVA-expressing L. pneumophila and OT-II cells. Although the presence of viable L. pneumophila was restricted to the lung and alveolar space, priming of L. pneumophila-specific OT-II cells occurred exclusively in the MLN and was independent of L. pneumophila T4SS. Thus, bacterial Ags have to be transported from the lung tissue to the draining lymph node, either by active transport via migratory APCs or by passive draining.

Despite the initial activation of OT-II cells in the MLN, we did not observe cytokine production by the primed cells in the MLN up to 10 d after priming. Interestingly, the development of effector functions by OT-II cells was entirely restricted to the airways, reminiscent of previous reports analyzing CD4 T cell priming upon pulmonary Aspergillus infection (49). The selective development of effector functions in the lung tissue correlates with the presence of live bacteria at this site, which might promote acquisition of T cell effector functions.

Although both initial activation and proliferation of OT-II cells were independent of L. pneumophila T4SS, OT-II cells primed in the absence of L. pneumophila T4SS failed to sustain proliferation and accumulation in the MLN, failed to efficiently migrate to the lung tissue, and most importantly, completely failed to differentiate into IFN-γ- and IL-17-producing effector cells. Hence, cytoplasmic pattern recognition, leading to the induction of a strong inflammatory response, was a prerequisite for Th1/Th17 effector T cell differentiation and for sustained proliferation and survival of the Ag-primed CD4 T cells.

In the absence of MyD88, L. pneumophila-specific Th17 responses were severely impaired; instead IL-4-producing L. pneumophila-specific CD4 T cells were induced. Interestingly, IL-4 and IFN-γ CD4 T cells appeared at the same time, with some IL-4/IFN-γ double producers, correlating with coexpression of T-bet and Gata-3 in OT-II cells. Previous work showed that the absence of MyD88 leads to impaired dendritic cell (DC) maturation and priming of Th1 responses, with unaffected Th2 responses and increased production of IgE. This led to the assumption that Th2 responses occur either by default in the absence of TLR signaling and IL-12 production by DCs or are actively induced via triggering of alternative PRRs (50). However, the absence of IL-12 does not appear to be decisive for Th2 induction and is not required for pathogen-induced Th1 polarization (51). In contrast, active induction of predominantly Th2 responses via cytosolic recognition of peptidoglycans by nod1 and nod2 was demonstrated in vivo studies (52, 53). Furthermore, L. pneumophila-derived peptidoglycans were shown to activate nod1 and nod2 (10, 54). Both nod1 and nod2 activate NF-κB-signaling pathways through the adapter serine-threonine kinase Rip2 and MAPK through CARD9. Because Th2-inducing signals might be mediated via the Rip2-signaling pathway, we assessed whether Th2 induction observed in MyD88−/− mice is abolished in the concomitant absence of Rip2. However, OT-II cells in Rip2/2 MyD88−/− recipient mice still expressed Gata-3 after i.n. L. pneumophila GFP-OVA infection (data not shown), suggesting that Th2 induction is mediated via CARD9, redundancy among both pathways, or via alternative pathways. Remarkably, most of the pathogens that were shown to induce Th2 responses in MyD88−/− mice were, like L. pneumophila, pathogens endowed with secretion systems, allowing PAMPs access to the host cell cytosol. In addition, they are all able to establish residence in a replication-permissive vacuole that does not fuse with lysosomes. Salmonella typhimurium, equipped with a type III secretion system, was shown to induce the Th2-dependent serum Ab isotypes IgG1, IgG2b, and IgA in MyD88−/− mice (55). Th2 responses were observed in MyD88−/− mice p.i. with Chlamydia muridarum (56), a bacterium expressing peptidoglycans (57) and possessing a type III secretion system. Moreover, the protozoan Leishmania major also triggers a Th2 response upon infection of MyD88−/− mice (58), and by escape from the phagosome into the cytosol (59), it gains access to cytosolic PRRs. Together with our results, these examples point to an active Th2-inducing signal that is triggered by pathogens that access the cytosolic compartment of host cells either directly or indirectly by delivery of PAMPs via secretion systems and that gets induced as soon as Th1-dominant signals mediated via TLRs are absent. Also in our L. pneumophila infection model, the absence of a dominant TLR-mediated Th1-inducing signal in MyD88−/− mice is likely to be responsible for the observed Th2 response, because caspase-1−−, IL-1R−−, and IL-18−− mice do not exhibit Th2 induction.

Previous studies documented a preferential induction of Th2 responses in the presence of low amounts of Ag in vitro (33). Low amounts of the TLR4 ligand LPS were shown to induce Th2 responses in an asthma model, whereas high amounts of LPS promoted Th1 induction (34). Interestingly, L. pneumophila-derived LPS is a poor TLR4 agonist in vitro and in vivo (60, 61), which, according to this hypothesis, favors a Th2 response. When using replication-incompetent thymidine auxotroph L. pneumophila, thereby normalizing the bacterial loads in WT and MyD88−/− mice, we still observed induction of a Th2 response. Hence, Th2 induction was not dependent on the amount of Ag or PAMPs, but rather the absence of TLR signaling. It remains to be determined whether the induction of a Th2 response in MyD88−/− mice has a biological role. However, IL-4 was suggested to protect from chronic inflammatory responses, and the highly increased pathogen load over more than a week in the lungs of MyD88−/− mice might approximate a chronic situation. Therefore, induction of a Th2 response in the absence of MyD88 might contribute to protection from tissue damage by MyD88-independent proinflammatory cytokines in chronic situations.

Taken together, our results suggest a negligible role for cytosolic pattern recognition of L. pneumophila-derived PAMPs in the initial activation of T cell responses in a WT host but a crucial role in the onset of inflammation and the subsequent instruction of effector cell differentiation. Furthermore, recognition of L. pneumophila by membrane-bound PRRs like TLRs is indispensable in guiding CD4 T cells into the appropriate Th1/Th17 lineage. However, in the absence of MyD88, cytosolic pattern recognition of L. pneumophila-derived PAMPs is crucial for CD4 T cell priming in the MLN. Hence, the integration of signals from various innate PRRs critically shapes the CD4 T cell response to a bacterial infection, which efficiently triggers innate-signaling pathways downstream of both membrane and cytosolic PRRs.
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