IL-1R signaling enables bystander cells to overcome bacterial blockade of host protein synthesis

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Edited by Ruslan Medzhitov, Yale University School of Medicine, New Haven, CT, and approved May 6, 2015 (received for review January 20, 2015)

The innate immune system is critical for host defense against microbial pathogens, yet many pathogens express virulence factors that impair immune function. Here, we used the bacterial pathogen *Legionella pneumophila* to understand how the immune system successfully overcomes pathogen subversion mechanisms. *L. pneumophila* replicates within macrophages by using a type IV secretion system to translocate bacterial effectors into the host cell cytosol. As a consequence of effector delivery, host protein synthesis is blocked at several steps, including translation initiation and elongation. Despite this translation block, infected cells robustly produce proinflammatory cytokines, but the basis for this is poorly understood. By using a reporter system that specifically discriminates between infected and uninfected cells within a population, we demonstrate here that infected macrophages produced IL-1α and IL-1β, but were poor producers of IL-6, TNF, and IL-12, which are critical mediators of host protection. Uninfected bystander cells robustly produced IL-6, TNF, and IL-12, and this bystander response required IL-1 receptor (IL-1R) signaling during early pulmonary infection. Our data demonstrate functional heterogeneity in production of critical protective cytokines and suggest that collaboration between infected and uninfected cells enables the immune system to bypass pathogen-mediated translation inhibition to generate an effective immune response.

L. pneumophila | IL-1 | IL-1R | TNF | type IV secretion system

Initiation of innate immune responses to microbial pathogens involves the direct recognition of pathogen-associated molecular patterns (PAMPs) by membrane-bound and cytosolic pattern recognition receptors (PRRs) in infected cells (1, 2). However, virulence factors of many pathogens interfere with essential immune signaling processes, including NF-κB and MAPK signaling and host protein synthesis (3–5). Such virulence factors would be expected to limit cell-intrinsic immune activation of infected cells. The mechanisms that enable the host to successfully overcome pathogen subversion of host cell processes remain poorly understood.

The Gram-negative bacterium *Legionella pneumophila* encodes a specialized Dot/Icm (for defect in organelle trafficking/intracellular multiplication) type IV secretion system (T4SS) that delivers bacterial effector proteins into host cells to facilitate its intracellular survival and replication (6–8). A subset of effector proteins, Lgt1, Lgt2, Lgt3, SidI, SidL, Pkn5, and Lpg1489, blocks host protein synthesis, in part by disabling elongation factors (9–13). Furthermore, host translational initiation is suppressed during infection due to diminished mTOR signaling (14). These activities result in a greater than 90% decrease in host translation in infected cells (13, 15). Nevertheless, *L. pneumophila* infection leads to robust production of many key protective proinflammatory cytokines (12, 16–19). Moreover, the presence of the T4SS paradoxically enhances cytokine production, suggesting that much of the host response against *L. pneumophila* is mediated by cytosolic sensing of bacterial ligands and virulence activities (13, 16, 17, 20).

How the host is able to mount a proinflammatory cytokine response when *L. pneumophila* potently blocks host translation remains unclear. At the population level, decreased host protein synthesis leads to preferential translation of the most abundant cytokine transcripts (14). At the single cell level, infected cells selectively synthesize IL-1α and IL-1β through a mechanism involving MyD88-dependent translational bypass (21). However, whether mechanisms that enable selective translation of IL-1 also apply to other key cytokines and immune effector proteins has not been determined. Alternatively, as a significant fraction of cells present during infection both in vitro and in vivo remain uninfected bystander cells, we considered the possibility that these uninfected bystander cells might respond to the presence of infection to produce cytokines instead (22). Here, by tracking immune responses in *L. pneumophila*-infected cells at the single cell level, we demonstrate that although infected cells receiving T4SS effectors synthesize IL-1α and IL-1β, they are poor producers of other key inflammatory proteins. Instead, bystander cells that have not received T4SS effectors are the primary producers of TNF, IL-6, IL-12, and the costimulatory molecule CD86 during both in vitro and in vivo infection. Importantly, lack of IL-1R signaling leads to reduced bystander cytokine production and increased bacterial burden in vivo, suggesting that the IL-1 released from infected cells enables bystander cells to produce proinflammatory cytokines. Our findings indicate that release of IL-1 by infected cells signals the presence of virulent infection, enabling the host to generate a robust innate immune response despite a pathogen-imposed translation block.

Results

Uninjected Cells Produce Key Inflammatory Mediators During in Vitro Infection. Infection with virulent *L. pneumophila* expressing a T4SS leads to an enhanced cytokine response despite bacterial inhibition of host translation. How this cytokine response is generated remains unclear. It is possible that directly infected macrophages possess cell-intrinsic mechanisms that enable

Significance

Pathogens use virulence factors to inhibit key immune cell functions and would be expected to impair immune responses to infection. However, immune responses are still generated against infection, suggesting that the immune system has evolved mechanisms for overcoming pathogenic activity. Here, we demonstrate that cells infected with *Legionella pneumophila* synthesize IL-1 despite a pathogen-imposed block in host translation, but are unable to produce other critical cytokines. IL-1 signaling allows uninfected bystander cells to produce protective cytokines. Our data thus demonstrate a key role for communication between infected and uninfected bystander cells in overcoming pathogenic activities. This mechanism of immune activation has broad significance for our understanding of how successful immune responses are generated against pathogens.

Author contributions: A.M.C., C.N.C., and S.S. designed research; A.M.C., C.N.C., H.T.N., and M.M.D. performed research; A.M.C., C.N.C., and S.S. analyzed data; and A.M.C., C.N.C., and S.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1501289112/-/DCSupplemental.

selective translation of cytokines. Alternatively, cytokines may be produced by bystander cells that are uninfected or have taken up bacteria that failed to translocate effectors (22). To determine whether T4SS-injected cells or uninjected bystander cells produce cytokines, we used a fluorescence-based system that detects the translocated *L. pneumophila* effector (RalF) fused to β-lactamase (BlaM) (22, 23). In the absence of BlaM activity, 409-nm excitation of the host cell-permeable BlaM fluorescent substrate CCF4-AM results in emission of green fluorescence at 518 nm. However, T4SS-translocated BlaM–RalF results in cleavage of CCF4-AM and a shift in emission to blue fluorescence at 447 nm. This system enables robust discrimination of infected and uninfected cells within tissues in vivo or in cultured cells in vitro (22).

We infected bone marrow-derived macrophages (BMDMs) with *L. pneumophila* encoding the BlaM–RalF reporter. As flagellin delivered by the T4SS into the host cell cytosol induces rapid cell death via NAIP5 inflammasome activation, we used flagellin-deficient *L. pneumophila* (Δ*flaA*) for these studies. Δ*flaA L. pneumophila* evade NAIP5 detection and replicate in C57BL/6 macrophages and mice (24–26), but still induce NLRP3 and caspase-11 inflammasome activation and IL-1 family cytokine secretion (16, 19, 27). Following infection, BMDMs were loaded with CCF4-AM and intracellularly stained for various cytokines at multiple timepoints postinfection. (Fig. 1 and Fig. S1). BMDMs infected with avirulent *L. pneumophila* lacking the Dot/Icm T4SS (Δ*dotA*) demonstrated no BlaM–RalF activity as expected. During Δ*flaA* infection, we found that TNF is rapidly produced, but T4SS-injected cells poorly produced TNF at all timepoints assayed (Fig. 1*A*). Instead, uninjected cells were the primary producers of TNF. Likewise, IL-6 was poorly produced by T4SS-injected cells and instead was primarily produced by uninjected cells (Fig. 1*B*). Similar findings were obtained for IL-12 and the costimulatory protein CD86 (Fig. S1 *A* and *B*). In contrast, IL-1α and IL-1β were robustly produced by both T4SS-injected cells and uninjected cells (Fig. 1 *C* and *D*), indicating that T4SS-injected cells selectively synthesize IL-1α and IL-1β despite the host translation block, consistent with recent findings (21). To determine whether the decreased cytokine production observed in T4SS-injected cells is due to decreased translation or transcription, we measured cytokine mRNA levels in sorted populations of T4SS-injected and uninjected cells (Fig. 1*E*). Both injected and uninjected cells displayed marked increases in *Il1a*, *Il1b*, *Tnf*, *and Il6* transcript levels relative to uninfected cells, with injected cells exhibiting significantly greater increases in cytokine transcript levels than uninjected cells, consistent with recent findings (21). Thus, the decreased production of immune proteins by injected cells is not due to a lack of transcriptional activation and most likely is due to impaired translation.

Seven *L. pneumophila* effectors are known to inhibit host translation (9, 12, 28). To determine if these effectors accounted for impaired cytokine production in injected cells, we infected BMDMs with Δ*5 L. pneumophila* lacking five of these effectors (Lgt1, Lgt2, Lgt3, SidI, and SidL) or the Δ7Δ*flaA* strain lacking all seven effectors (Δ*5* additionally deleted for Pkn5 and Lpg1489) (12, 13). At 4 h postinfection, cells injected by the Δ*5* or Δ7Δ*flaA* strains produced substantially more TNF than WT- or Δ*flaA*-injected cells, with an increase both in the frequency of TNF-producing cells and the TNF geometric mean fluorescence intensity (gMFI) (Fig. S2 *A* and *D*). IL-1α and IL-1β production was also increased in cells injected with the Δ*5* or Δ7Δ*flaA* strains compared with controls (Fig. S2 *G–I*). By 16 h postinfection, however, there was no difference in the frequency of TNF-producing injected cells with either the Δ*5* or Δ7Δ*flaA* strains compared with the parental strains, although the TNF gMFI was significantly increased in cells injected by the Δ7Δ*flaA* strain compared with the Δ*flaA* strain (Fig. S2 *B* and *E*). The frequency of IL-6–producing injected cells was similarly unaffected by the presence or absence of these effectors (Fig. S2 *C* and *F*). These data indicate that early in infection, these bacterial effectors suppress cytokine translation, whereas at later timepoints, additional host and/or bacterial mechanisms inhibit cytokine translation in injected cells. These findings would be consistent with previous findings showing that the Δ*5* and Δ7Δ*flaA* strains still partially inhibit host protein synthesis, indicating that yet additional bacterial or host mechanisms suppress host translation. Taken together, these data suggest that although productively infected cells selectively translate IL-1α and IL-1β, they are unable to synthesize TNF, IL-6, IL-12, and CD86. Instead, bystander cells are the primary producers of these key inflammatory proteins.

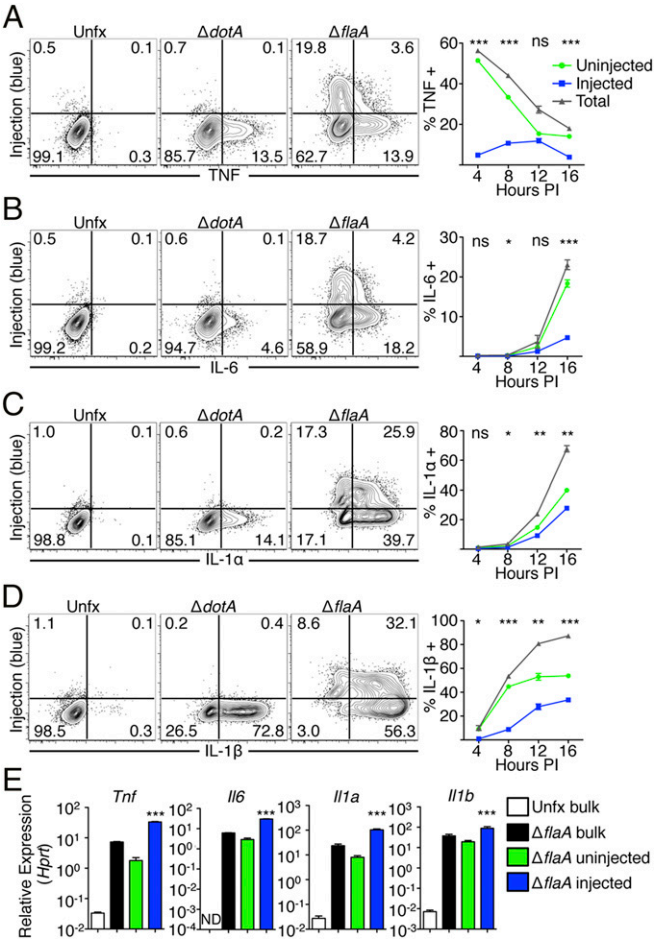

Fig. 1. T4SS-injected and uninjected macrophages produce different cytokines in vitro. (*A–D*) Bone marrow-derived macrophages (BMDMs) were infected with Lp02 strains of *L. pneumophila* for 4, 8, 12, or 16 h. BMDMs were treated with brefeldin A and monensin, loaded with CCF4-AM, fixed, and stained with antibodies against TNF (*A*), IL-6 (*B*), IL-1α (*C*), and IL-1β (*D*). Plots show cytokine production at 16 h postinfection (PI). Line graphs show Δ*flaA*-infected macrophages where total frequency of cytokine-producing cells as well as the contribution of injected and uninjected cells to cytokines are shown. NS, not significant, $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$ as determined by Student's *t* test between uninjected and injected cells at various times PI. (*E*) BMDMs were infected with Lp02 ΔflaA *L. pneumophila* for 8 h. Cells were then loaded with CCF4-AM and sorted based upon cleavage of the dye or were collected after going through the sorter without separating the cells based on dye cleavage (bulk). Sorted cells were then lysed and relative abundance of transcripts for proinflammatory cytokines were assayed using RT-PCR. $***P < 0.0005$ as determined by Tukey posttest between uninjected and injected samples. All graphs are $n = 3$ wells per condition per timepoint. All graphs show mean ± SEM and are representative of three independent experiments.

Bystander Alveolar Macrophages and Neutrophils Produce TNF During *L. pneumophila* Infection. Our in vitro findings indicate that infected cells are unable to produce many key protective cytokines. Instead, these cytokines are primarily generated by bystander macrophages. In vivo, multiple cell populations exist, and distinct immune populations may possess alternative mechanisms for overcoming a pathogen-induced translation block. We therefore examined TNF production during pulmonary infection with Δ*flaA* or Δ*dotA L. pneumophila*. At 24 h postinfection, increased levels of bacteria are present during Δ*flaA* infection, whereas Δ*dotA* bacteria do not replicate. However, cytokine responses to Δ*flaA* bacteria do not require increased bacterial load or bacterial replication, suggesting that the cytokine response is primarily driven by cytosolic sensing of T4SS activity (16, 17). We first focused on airway-resident alveolar macrophages and recruited neutrophils, as they are the primary cell types that receive T4SS effectors during pulmonary infection (22). Following intranasal infection with Δ*flaA L. pneumophila*, there was a significant increase in both the percentage and total numbers of TNF-producing airway alveolar macrophages and neutrophils (Fig. 2), consistent with previous findings showing that cytosolic immune sensing of T4SS activity is required for maximal TNF production (16, 17). However, we observed that T4SS-injected alveolar macrophages and neutrophils did not produce TNF. Instead, TNF was produced almost exclusively by uninjected alveolar macrophages and neutrophils (Fig. 2).

In contrast, both injected and uninjected alveolar macrophages produced IL-1α and IL-1β, in agreement with our in vitro findings (Fig. S3 *A* and *B*). PBS vehicle control and avirulent Δ*dotA* infection yielded no significant increase in TNF-producing alveolar macrophages or neutrophils (Fig. 2), corroborating previous findings that TNF is undetectable in the bronchoalveolar lavage (BAL) fluid or serum from similarly treated mice (16, 17). Similar results were obtained with alveolar macrophages and neutrophils isolated from lung tissue (Fig. S3 *E–H*). These data indicate that although cytosolic immune sensing of T4SS-translocated bacterial products is critical to elicit TNF production, TNF is produced by bystander alveolar macrophages and neutrophils that have not received T4SS-translocated substrates.

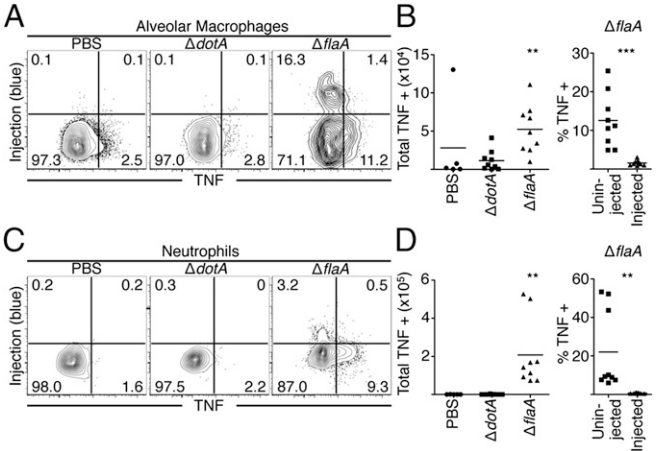

Fig. 2. Bystander alveolar macrophages and neutrophils produce TNF during *L. pneumophila* infection. Mice were infected and cells were harvested from the BAL fluid and lung tissue, treated with BFA, loaded with CCF4-AM, stained, and fixed and stained with an antibody against TNF. (*A–D*) Representative plots from alveolar macrophages (*A*) and neutrophils (*C*) collected from the BAL fluid 24 h PI and quantified in *B* and *D*. Graphs show individual mice and mean from three pooled independent experiments. Graphs on far right show the relative contribution of uninjected and injected cells to TNF production from Δ*flaA*-infected mice. $n = 2–3$ mice per group per experiment. Student's *t* tests were performed for total TNF-producing cells between Δ*dotA*- and Δ*flaA*-infected mice. $^{**}P < 0.005$, $^{***}P < 0.0005$.

Bystander Inflammatory Monocytes and Dendritic Cells Are the Primary Producers of TNF and CD86 During *L. pneumophila* Infection. Inflammatory monocytes and conventional dendritic cells (DCs) are also recruited to the lung during *L. pneumophila* infection. In contrast to alveolar macrophages and neutrophils, these cell populations are not productively infected and do not receive T4SS effectors (18, 22). Even so, inflammatory monocytes and DCs were the primary cell types that produced TNF during Δ*flaA L. pneumophila* infection (Fig. 3 *A* and *B*). In contrast, inflammatory monocytes and DCs from Δ*dotA*-infected mice did not increase TNF production compared with cells from PBS-treated mice. Inflammatory monocytes and DCs also substantially increased CD86 expression during Δ*flaA L. pneumophila* infection compared with PBS or Δ*dotA* infection and were the primary cell types expressing CD86 (Fig. 3 *C* and *D*). Alveolar macrophages and neutrophils did not increase CD86 expression during Δ*flaA* infection (Fig. S3 *C* and *D*). These data indicate that although inflammatory monocytes and DCs are not productively infected and do not receive T4SS-translocated products, they are the primary cell types that express TNF and CD86 during virulent *L. pneumophila* infection.

IL-1 Signaling Is Required for Bystander Immune Cells to Produce Cytokines and CD86. Maximal immune responses to *L. pneumophila* require cytosolic sensing of T4SS activity (16), yet our data indicate that the majority of cytokine- and CD86-producing cells are bystander innate immune cells that have not encountered T4SS effectors. Given that T4SS-injected cells selectively synthesize IL-1α and IL-1β, we hypothesized that IL-1α and IL-1β released by infected cells would instruct bystander cells to produce inflammatory cytokines, as IL-1α and IL-1β can elicit cytokine expression in other systems (29, 30). Notably, IL-1R signaling is critical for host defense against *L. pneumophila*, although its contribution has been primarily attributed to eliciting neutrophil-attracting chemokines and recruitment of neutrophils (12, 18, 19, 31). Following intranasal infection of WT or *Il1r1*$^{-/-}$ (IL-1R$^{-/-}$) mice with Δ*flaA L. pneumophila*, equivalent numbers of alveolar macrophages in the lung and airway space were injected by *L. pneumophila* in WT and IL-1R$^{-/-}$ mice; however, as IL-1R$^{-/-}$ mice fail to recruit neutrophils, the total number of injected neutrophils is decreased in these mice (Fig. S4). Despite carrying an increased bacterial load (19), IL-1R$^{-/-}$ mice exhibited significantly reduced percentages and total numbers of TNF-producing alveolar macrophages, neutrophils, DCs, and inflammatory monocytes compared with WT mice (Fig. 4 and Fig. S5 *A* and *B*). Although a percentage of IL-1R$^{-/-}$ DCs and inflammatory monocytes still produced TNF, it was only minimally higher than the basal percentage of TNF-positive cells in WT or IL-1R$^{-/-}$ mice infected with Δ*dotA* bacteria (Fig. S6). Furthermore, TNF and IL-12p40 levels in the BAL fluid of IL-1R$^{-/-}$ mice were significantly reduced compared with WT mice (Fig. 4*E*). IL-1R$^{-/-}$ mice also exhibited significantly decreased percentages and total numbers of CD86-expressing inflammatory monocytes and DCs compared with WT controls (Fig. S5 *C* and *D*). In contrast, IL-6 levels were unaffected, indicating that IL-1R signaling is not required for all bystander cell responses (Fig. 4*E*). Finally, antibody-mediated neutralization of IL-1α and/or IL-1β before infection significantly decreased levels of TNF and IL-12p40, but not IL-6, with neutralization of IL-1α and IL-1β together having the greatest effect (Fig. S5*E*). Overall, these data indicate a crucial role for IL-1R signaling in optimal production of the proinflammatory cytokines TNF and IL-12p40 as well as the costimulatory molecule CD86 by bystander immune cells during pulmonary *L. pneumophila* infection.

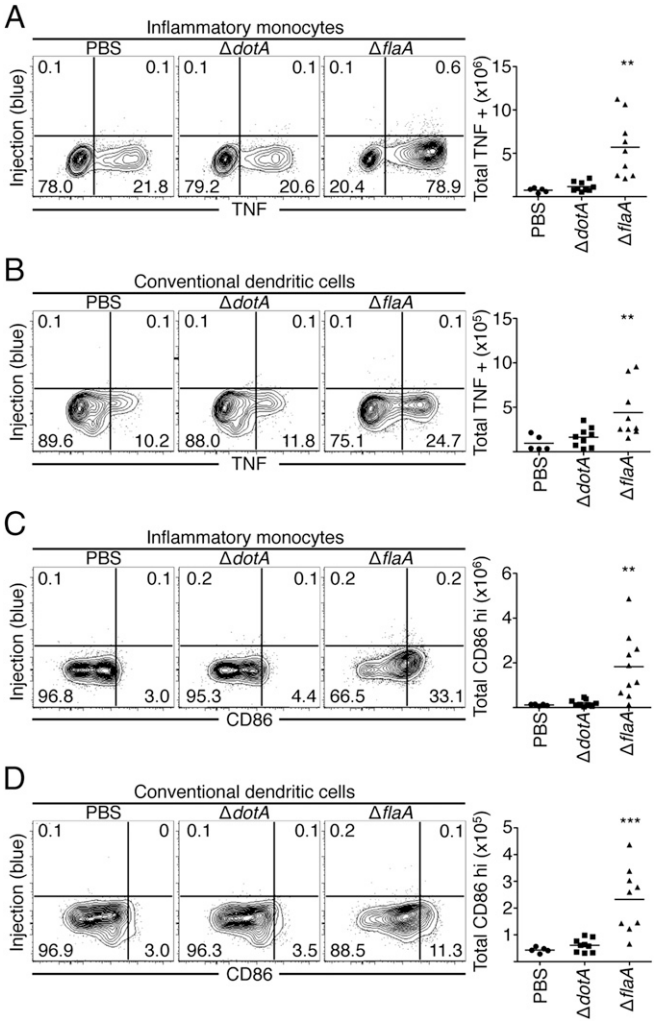

Fig. 3. Bystander inflammatory monocytes and conventional dendritic cells produce TNF and express CD86 during infection. Cells were harvested from the lung tissue of mice, treated with BFA, loaded with CCF4-AM, stained, and fixed and stained with an antibody against TNF (*A* and *B*) or CD86 (*C* and *D*). Representative plots are shown. Graphs show individual mice and mean from three pooled independent experiments. Student's *t* tests were performed between Δ*dotA*-and Δ*flaA*-infected mice. $^{**}P < 0.005$, $^{***}P < 0.0005$.

In contrast to our in vivo findings, we observed equivalent levels of TNF and IL-6 in IL-1R$^{-/-}$ and WT BMDMs during in vitro infection, suggesting that IL-1R signaling was not required for bystander responses in these cells (Fig. S7 *A* and *B*). Consistently, BMDMs treated with recombinant IL-1α and/or IL-1β did not produce TNF or IL-6, in contrast to LPS treatment, which robustly induced TNF and IL-6 (Fig. S7*C*). This lack of responsiveness to IL-1 was not due to the inability of IL-1α or IL-1β to induce responses, as treatment of the NIH/3T3 fibroblast line elicited robust IL-6 production (Fig. S7*D*). These data suggest that signals other than IL-1 are required for induction of bystander cytokine responses in BMDMs during in vitro infection, consistent with the view that BMDMs express very low levels of IL-1R and are poorly responsive to IL-1 (32, 33).

Other key innate immune cell types responded to IL-1 treatment. Indeed, bone marrow-derived DCs up-regulated CD86, TNF, and IL-6 in response to IL-1α and -β (Fig. S8 *A–D*). Moreover, alveolar macrophages from WT mice also produced TNF upon IL-1α and -β treatment, whereas IL-1R$^{-/-}$ alveolar macrophages did not (Fig. S8 *E–G*). In contrast, inflammatory monocytes isolated from bone marrow did not produce TNF or CD86 in response to IL-1α and -β (Fig. S7 *E–H*). Inflammatory monocytes produced TNF but not CD86 in response to LPS, suggesting that additional signals are required for inflammatory monocytes to express CD86 (Fig. S7*G*). Together, these data indicate that DCs and alveolar macrophages are likely to respond to IL-1 signaling in a cell-intrinsic manner. Other cell types may require either additional signals to produce cytokines or require an alternative signal produced by an intermediate cell in response to IL-1.

Discussion

Infection with *L. pneumophila* leads to a robust proinflammatory cytokine response that requires cytosolic sensing of T4SS activity (13, 16). This findng is paradoxical, as a number of T4SS effectors inhibit host protein synthesis (9–12, 14, 15, 20). We therefore sought to determine how this robust cytokine response is generated. Our data indicate that cells targeted by T4SS effectors selectively synthesize IL-1α and IL-1β, but poorly produce the key immune proteins TNF, IL-6, IL-12, and CD86 (Figs. 1–4). Instead, bystander cells produce the majority of these immune signals in vitro and in vivo. Furthermore, IL-1R signaling is required for bystander cells to produce these immune proteins in vivo. Thus, our data suggest that collaboration between infected cells and bystander cells overcomes the pathogen-imposed translation block by enabling bystander cells to mount an inflammatory cytokine response.

Our findings indicate that uninfected bystander cells play a critical role in host defense by producing immune effector proteins in response to direct or indirect sensing of IL-1 and additional signals released by infected cells. This mechanism provides multiple possible benefits for enabling robust antimicrobial

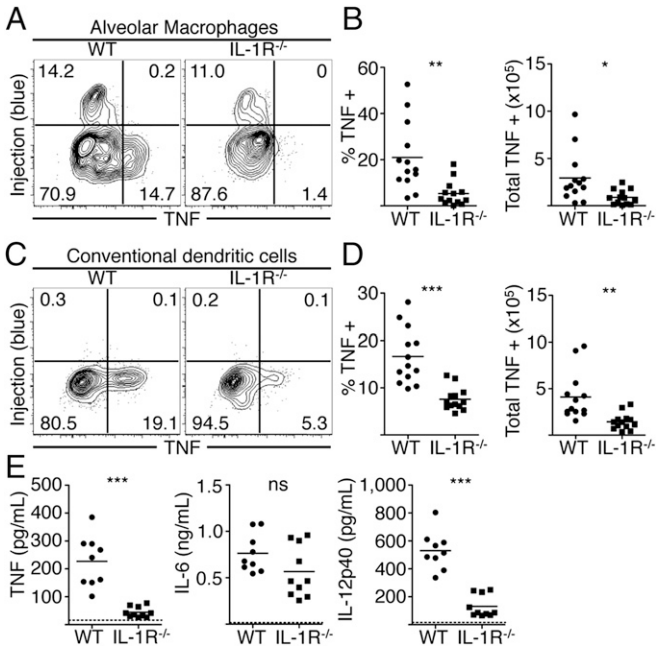

Fig. 4. IL-1 signaling induces cytokine production by bystander cells. Cells were harvested from the lungs of infected WT or *Il1r1*$^{-/-}$ (IL-1R$^{-/-}$) mice, treated with BFA, loaded with CCF4-AM, stained, fixed and then stained with an antibody against TNF. Representative plots from alveolar macrophages (*A* and *B*) and conventional DCs (*C* and *D*) are shown. Graphs show individual mice and mean pooled from four independent experiments. (*E*) BAL fluid was collected from mice and cytokine levels were measured by ELISA. Graphs show individual mice and mean pooled from three independent experiments. n = 2–4 mice per group. Student's *t* tests were performed between pooled groups. NS, not significant, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

immune defense. First, it allows for initiation of an immune response despite pathogen inhibition of host translation. Second, it may be a strategy for avoiding inappropriate responses to avirulent bacteria, as an inflammatory response against pathogens would be generated only when a second signal, such as IL-1, is specifically released upon sensing of virulence activity (34, 35). Third, bystander cytokine production may provide a means of amplifying early immune signals, allowing for a more rapid and robust response. Bystander activation is likely to be a common strategy used by the immune system for overcoming pathogen subversion, and multiple mechanisms may exist for activating various bystander cell types in the context of different pathogens (36–41). Notably, during influenza A virus infection, IL-1R signaling activates bystander DCs to enable successful priming of naïve $CD8^+$ T cells (42). This study, along with our finding that uninfected bystander cells up-regulate the costimulatory molecule CD86 (Fig. 3 and Fig. S5), has implications for understanding how a T-cell response is generated during *L. pneumophila* infection. Bystander cells may serve as key antigen presenting cells, perhaps following uptake of dead or dying infected cells (43, 44).

Our findings also elucidate another critical function for IL-1 in early innate immune defense. In addition to the well-established role of IL-1R signaling in eliciting expression of neutrophil-attracting chemokines and subsequent neutrophil recruitment to the site of infection (12, 18, 19, 31), our data indicate that IL-1R signaling mediates cytokine production by bystander immune cells (Fig. 4 and Fig. S5). Immune signals other than IL-1 also likely contribute during in vivo *L. pneumophila* infection, as IL-1R signaling is required for maximal production of TNF and IL-12, but is dispensable for IL-6 production. Other signals contribute in vitro, as IL-1R signaling is not required for bystander cytokine responses in BMDMs. The nature of these other immune signals and whether they can compensate for the absence of IL-1 in certain infection settings remains to be determined.

It is intriguing that infected cells effectively translate IL-1α and IL-1β, but not other key inflammatory proteins. IL-1α and IL-1β differ from other secreted cytokines, because they are translated by cytosolic ribosomes as precursors that are unconventionally secreted in an inflammasome-dependent manner (45, 46). MyD88 and host modulation of mTOR signaling participate in the selective translation of proinflammatory cytokines during *L. pneumophila* infection (14, 21) Whether MyD88 and mTOR signaling influence cytokine production in directly infected cells or bystander cells remains to be determined. Previous studies have suggested that mRNA abundance determines which transcripts are selectively translated when translation is suppressed in infected cells (14). Consistent with such a model, our data indicate a slight increase in the total abundance of translated transcripts (*Il1a* and *Il1b*) compared with transcripts that are not translated (*Il6* and *Tnf*). It remains to be determined, however, whether mRNA abundance fully explains the ability of injected cells to translate IL-1α and IL-1β but not other cytokines.

We found that the inability of injected cells to produce cytokines is partly due to effector activity, as cells injected by *L. pneumophila* strains lacking the translation-inhibiting effectors produced increased levels of TNF, IL-1α, and IL-1β early during infection (Fig. S2). However, at later times, cells injected by these mutants produced little TNF and IL-6. Why injected cells are unable to produce TNF and IL-6 later in infection is unclear. Multiple mechanisms may act in concert to impair host translation at later timepoints, including yet-to-be-identified effectors that inhibit host translation or host-driven responses that suppress mTOR signaling (14).

Because IL-$1R^{-/-}$ mice have impaired bystander responses during in vivo infection, we investigated whether IL-1R signaling was sufficient to induce TNF, IL-6, or CD86 expression in various innate immune cell types. Previous studies demonstrated that IL-1 stimulation of both mouse and human immune cells induces TNF production (33, 47), and IL-1 treatment induces CD86 expression on lung DCs (42). We found that alveolar macrophages and bone marrow-derived DCs responded to IL-1 stimulation ex vivo (Fig. S7). The amount of TNF and CD86 elicited by IL-1, however, is moderate compared with LPS treatment or the levels attained during in vivo infection. IL-1 treatment was insufficient for TNF or CD86 expression in inflammatory monocytes ex vivo, although IL-1R signaling is required for these cells to express TNF and CD86 during in vivo infection. We therefore conclude that IL-1R signaling is necessary for robust bystander responses during in vivo infection, but is only partially sufficient. We hypothesize that additional host and/or microbial signals work in concert with IL-1 to drive bystander responses, and some cell types may indirectly respond to IL-1 or other factors via signals produced by an intermediary cell. These other signals may be required for bystander responses in BMDMs and may account for IL-6 production and the remaining TNF made by IL-$1R^{-/-}$ inflammatory monocytes during in vivo infection.

In conclusion, we have identified a system used by the innate immune system to ensure generation of proinflammatory cytokines during infection with a bacterial pathogen. Our results demonstrate the existence of heterogeneity in production of critical protective cytokines by innate immune cells during the early response to bacterial infection. These findings define a collaboration between infected and uninfected cells that enables the immune system to bypass pathogen-mediated inhibition of protein synthesis to generate a robust immune response against *L. pneumophila*. As a variety of pathogens would be expected to disable cytokine production in directly infected cells, uninfected bystander cells may be required to carry out and amplify the early innate signals that eventually confer protective immunity.

Materials and Methods

Ethics Statement. All experiments performed in this study were done in accordance with the Animal Welfare Act and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (48). The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (Protocols 803465, 803459, 804714, and 804928).

Bacterial Strains and Plasmids. All experiments used *L. pneumophila* serogroup 1 strains. For in vitro studies, macrophages were infected with Lp02 (*rpsL*, *hsdR*, and *thyA*), a thymidine auxotroph derived from strain Lp01, Lp02 Δ*dotA*, Lp02 Δ*flaA*, Lp02 Δ*lgt1*Δ*lgt2*Δ*lgt3*Δ*sidI*Δ*sidL* (WTΔ*5*), or Lp02 Δ*flaA*Δ*lgt1*Δ*lgt2*Δ*lgt3*Δ*sidI*Δ*sidL*Δ*pkn5*Δ*lpg1489* (Δ*7*Δ*flaA*) (12, 13). For in vivo studies, mice were infected with JR32-derived (*rpsL* and *hsdR)* Δ*dotA* or Δ*flaA* isogenic mutant strains. All strains harbored the pSS128 plasmid encoding an M45-tagged β-lactamase–RalF fusion protein (22). For in vitro and in vivo studies, *L. pneumophila* was cultured on charcoal yeast extract agar containing 6.25 μg/mL chloramphenicol for 48 h at 37 °C before infection (19, 49–51).

Mice. C57BL/6 and IL-$1R^{-/-}$ (*Il1r1*$^{-/-}$) mice were purchased from The Jackson Laboratories. Mice were maintained in accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. Please refer to *SI Materials and Methods* on infection of mice, cell culture, isolation of monocytes and alveolar macrophages, flow cytometry and cell sorting, IL-1 stimulation, RT-PCR, and ELISA. Differences were considered significant if the *P* value was <0.05.

ACKNOWLEDGMENTS. We thank Russell Vance for providing the WTΔ*5* and Δ*7*Δ*flaA L. pneumophila* strains, Mike Askenase in Yasmine Belkaid's laboratory for providing protocols on inflammatory monocyte isolation and ex vivo stimulation, members of the Brodsky laboratory for helpful discussions, and Igor Brodsky for critical reading of the manuscript. This work was supported in part by National Institutes of Health Grants K99/R00AI087963 (to S.S.) and T32GM007229 (to A.M.C.), American Lung Association Grant RG-268528-N (to S.S.), University of Pennsylvania University Research Foundation (S.S.), an Institute for Immunology Pilot Award (to S.S.), American Heart Association Grant 13BGIA14780070 (to S.S.), and the Roy and Diana Vagelos Scholars Program in Molecular Life Sciences (M.M.D.). This material is based upon work supported by the National Science Foundation under Grant DGE-0822 (to C.N.C., a graduate research fellowship).

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