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Authors

O'Donnell, Hope
Pham, Oanh H
Li, Lin-xi
et al.

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Toll-like receptor and inflammasome signals converge to amplify the innate bactericidal capacity of T helper-1 cells

Hope O'Donnell[1,2], **Oanh H. Pham**[1], **Lin-xi Li**[1], **Shaikh M. Atif**[1], **Seung-Joo Lee**[1], **Marietta M. Ravesloot**[1], **Jessica L. Stolfi**[1], **Sean-Paul Nuccio**[3], **Petr Broz**[4], **Denise M. Monack**[4], **Andreas J. Baumler**[3], and **Stephen J. McSorley**[1]

[1]Center for Comparative Medicine, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California Davis, Davis, CA 95616, USA

[2]Microbiology, Immunology, and Cancer Biology Graduate Program, University of Minnesota Medical School-Twin Cities, Minneapolis, MN 55455, USA

[3]Department of Medical Microbiology and Immunology, School of Medicine, University of California Davis, Davis, CA 95616, USA

[4]Department of Microbiology and Immunology, School of Medicine, Stanford, CA 94305, USA

Summary

T cell effector functions can be elicited by non-cognate stimuli, but the mechanism and contribution of this pathway to the resolution of intracellular-macrophage infections has not been defined. Here we have shown that $CD4^+$ T helper-1 (Th1) cells can be rapidly stimulated by microbe-associated molecular patterns (MAMPs) during active infection with *Salmonella* or *Chlamydia*. Further, maximal stimulation of Th1 cells by lipopolysaccharide (LPS) did not require T cell-intrinsic expression of toll-like receptor-4 (TLR4), interleukin-1 receptor (IL-1R), or interferon-γ receptor (IFN–γR), but instead required the adaptor protein Myd88, IL-18R, and IL-33R. Innate stimulation of Th1 cells also required host expression of TLR4 and inflammasome components that together increased serum concentrations of IL-18. Finally, the elimination of non-cognate Th1 cell stimulation hindered the resolution of primary *Salmonella* infection. Thus, the in vivo bactericidal capacity of Th1 cells is regulated by the response to non-cognate stimuli elicited by multiple innate immune receptors.

Introduction

Pathogen-specific lymphocytes recirculate at low frequency between the blood and secondary lymphoid tissues and undergo rapid expansion in response to infection (Kwok et al., 2012; Moon et al., 2007). As clonal expansion occurs, responding T cells integrate local instructional stimuli to acquire effector functions tailored to combat different pathogen types (Obar and Lefrancois, 2010; Zhu et al., 2010). The expansion and functional maturation of individual T cell clones is tightly regulated by pathogen-specific T cell receptors (TCRs) that recognize microbial peptides in the context of host Major Histocompatibility Complex (MHC) molecules. Thus, the adaptive immune response to infection produces a large

*Correspondence should be addressed to Stephen McSorley, Tel: (530) 752 3414, Fax: (530) 752 7914 sjmcsorley@ucdavis.edu.

population of antigen-specific effector T cells with appropriate functional activities to combat invading microbes.

Although the initial activation and expansion of pathogen-specific T cells is controlled by TCR ligation, the subsequent signals for inducing T cell effector functions are incompletely understood. In a non-infectious context, the elicitation of effector functions by tissue-resident $CD4^+$ T cells requires T cell receptor (TCR) recognition of cognate antigen presented by local antigen presenting cells (McLachlan et al., 2009). However, a lower threshold for stimulating activated effector T cells may be advantageous when confronting a replicating pathogen, especially one that can manipulate host MHC expression (Griffin and McSorley, 2011). Indeed, inflammatory cytokines cause non-cognate stimulation of effector $CD8^+$ T cells, notably interleukin-12 (IL-12) and IL-18 (Beadling and Slifka, 2005; Berg et al., 2002; Freeman et al., 2012). During bacterial infections, the production of inflammatory cytokines can be initiated by host recognition of conserved molecular patterns via multiple innate immune receptors (Broz and Monack, 2011). Thus, bacterial flagellin can efficiently drive non-cognate stimulation of $CD8^+$ memory T cells in a process that involves dendritic cell sensing of cytosolic flagellin by nucleotide binding domain and leucine rich repeat CARD domain-containing protein 4 (NLRC4) (Kupz et al., 2012). However, the role of toll-like receptor (TLR) and inflammasome signaling in the elicitation of T cell effector functions is currently unclear. Such non-cognate stimulation pathways may allow T cell effector functions to be induced rapidly in an inflammatory context and provide an evolutionary advantage for the host in combating bacterial pathogens.

The efferent phase of the $CD4^+$ Th1 cell response to an intra-macrophage pathogen has the potential to be relatively non-specific, since it consists of macrophage activation by locally produced interferon-gamma (IFN-γ). Although cytokine secretion may be restricted to the synapse during cognate (antigen receptor agonist) stimulation, $CD4^+$ Th1 cells can activate macrophages in the absence of cognate stimuli and also provide cross-protection against unrelated co-infecting microbes (Mackaness, 1964; Muller et al., 2012; Poo et al., 1988). While Th1 cell secretion of IFN-γ can be induced by cognate antigen and major histocompatibility complex (MHC) class-II presented on infected phagocytes, it can also occur in the presence of cytokines (Robinson et al., 1997; Takeda et al., 1998) or TLR ligation (Caramalho et al., 2003; Reynolds et al., 2010). However, the signals that drive non-cognate stimulation of $CD4^+$ Th1 cells and the contribution of this pathway to bacterial clearance have not been clearly defined in vivo.

Here, we have examined the mechanism and contribution of non-cognate T cell stimulation to the resolution of intra-macrophage infection. Expanded T-bet^+ $CD4^+$ T cells in *Salmonella*-, and *Chlamydia*-infected mice were induced to secrete IFN-γ by brief in vivo exposure to ligands for TLR2, 4, and 5, and this required T cell-intrinsic expression of the adaptor protein Myd88 and IL-18R, as well as an unexpected role for IL-33R in this amplification of Th1 cell responses. Generating an inflammatory environment favoring non-cognate stimulation of Th1 cells required host expression of TLR and inflammasome components, which together enhanced concentrations of circulating IL-18. $CD8^+$ T cells were able to respond in a similar manner, but made a smaller contribution to bacterial clearance. Mice lacking T cell-intrinsic expression of Myd88 had impaired ability to control primary *Salmonella* infection, demonstrating the importance of non-cognate responses to the resolution of an intra-macrophage infection. Overall, these data show that non-cognate stimulation of T cells can occur in response to innate inflammatory cues and contribute to defense against intra-macrophage pathogens.

Results

CD4$^+$ and CD8$^+$ T cells in infected mice can secrete IFN-γ in response to innate receptor stimuli

Salmonella infection of C57BL/6 mice induces the expansion of splenic CD44^{Hi} CD4$^+$ and CD8$^+$ T cell populations that persist as a major fraction of the T cell pool until bacterial clearance occurs, approximately 5–8 weeks later (Fig. 1A) (Srinivasan et al., 2004). The majority of CD4$^+$ T cells responding to *Salmonella* infection expressed the transcription factor T-bet (Fig. 1B), consistent with a requirement for Th1 cells in the resolution of intra-macrophage infections (Griffin and McSorley, 2011). A small population of CD4$^+$ (<5%) or CD8$^+$ (<2%) T cells in the spleen of *Salmonella*-infected mice were found to be actively secreting IFN-γ and these were part of the expanded CD44^{Hi} population, and undetectable in uninfected mice (Figure 1C, no stim). However, intravenous (IV) injection of infected mice with ultrapure lipopolysaccharide (LPS) induced IFN-γ secretion from approximately 30–50% of CD4$^+$ T cells and 5–20% of CD8$^+$ T cells within four hours, while a response to innate receptor stimuli was not detected in uninfected recipients (Figure 1C). Among CD4$^+$ T cells, LPS-induced IFN-γ production was confined to T-bet$^+$ cells, and typically 50–70% of all T-bet$^+$ CD4$^+$ T cells participated in this innate response at the peak of infection (Fig. 1D and E). As *Salmonella* infection resolves around day 35 (Srinivasan et al., 2004), the proportion of T-bet$^+$ CD4$^+$ T cells able to respond to innate stimuli correspondingly decreased (Fig. 1E). However, a small population (5–10%) of T-bet$^+$ CD4$^+$ T cells retained the ability to respond rapidly to innate stimuli for at least 24 weeks after primary infection (Fig. 1E). A similar response was detected among activated CD8$^+$ T cells, but with a lower magnitude than CD4$^+$ T cells at each time point (Fig. 1E). There were no other major splenic cell populations that produced IFN-γ in response to innate stimuli (Fig. S1), indicating that CD4$^+$ and CD8$^+$ T cells are the major IFN-γ-producing cells in this model.

To determine whether innate amplification of CD4$^+$ Th1 cell effector function was a process unique to *Salmonella* infection, we examined C57BL/6 mice infected with *Chlamydia muridarum*, another common intra-macrophage pathogen (Farris and Morrison, 2011). T-bet$^+$ CD4$^+$ T cells in the spleen of C57BL/6 mice infected with *Chlamydia muridarum* produced IFN-γ rapidly in response to LPS stimulation (Fig. 2A and B). LPS injection also caused a small population of CD8$^+$ T cells to secrete IFN-γ (Fig. 2B). Similar to the *Salmonella*-infection model, little IFN-γ secretion was detectable in CD4$^+$ T cells of uninfected mice injected with LPS, or in *Chlamydia*-infected mice prior to LPS injection (Fig. 2A and B). Next, we examined whether other TLR ligands had the capacity to induce IFN-γ production from T-bet$^+$ CD4$^+$ T cells by injecting *Salmonella*-infected mice with LPS (recognized by TLR4), flagellin (TLR5), Pam3CSK4 (TLR1 and TLR2), CpG DNA (TLR9), or Imiquimod (TLR7). Injection of LPS, flagellin, or Pam3CSK4 amplified the production of IFN-γ from T-bet$^+$ CD4$^+$ T cells in *Salmonella*-infected mice, with LPS acting as the most effective inducer (Fig. 2C and D). A small percentage of CD8$^+$ T cells also produced IFN-γ in response to each of these stimuli (Fig. 2D). In contrast, the injection of CpG DNA or Imiquimod failed to induce IFN-γ production over baseline production detected in CD4$^+$ and CD8$^+$ T cells from *Salmonella*-infected mice (Fig. 2C and D). LPS stimulation of T-bet$^+$ CD4$^+$ and CD44$^+$ CD8$^+$ T cells was greatest when 25 micrograms of LPS was used, however, a rapid innate response was also detectable when as little as 100 nanograms of LPS was injected (Fig. 2E). Together, these results demonstrate that innate stimulation of CD4$^+$ and CD8$^+$ T cells can occur rapidly in response to a variety of microbial stimuli and that this capability is a common feature of the immune response to intra-macrophage infections.

T cells require expression of Myd88, but not toll-like receptor-4 (TLR4), for innate amplification

Mixed bone marrow (BM) chimeric mice were generated to define the T-cell intrinsic requirements for innate amplification of $CD4^+$ and $CD8^+$ T cell effector functions. Congenically-marked ($CD90.2^+$ $CD45.1^+$) mice were irradiated and reconstituted with a 1:1 mixture of BM from wild-type ($CD90.1^+$ $CD45.2^+$) and a variety of gene-deficient ($CD90.2^+$ CD45.2+) mice (Fig. 3A), allowing direct comparison of wild-type and gene-deficient T cells within a single *Salmonella*-infected host. As expected, $CD4^+$ and $CD8^+$ T cells in *Salmonella*-infected, non-chimeric, TLR4-deficient mice were unable to produce IFN-γ in response to innate stimulation with LPS (Fig. S2). However, in *Salmonella*-infected TLR4-deficient:wild-type chimeras, both wild-type and TLR4-deficient $CD4^+$ and $CD8^+$ T cells responded equally well to LPS stimulation (Fig. 3B and Fig. S3A). Next, we utilized mixed BM chimeras to examine the requirement for Myd88, an essential component of most TLR and IL-1-like receptor signaling. $CD4^+$ and $CD8^+$ T cells lacking expression of Myd88 were unable to respond to LPS injection, while wild-type $CD4^+$ and $CD8^+$ T cells in the same mouse generated robust IFN-γ responses (Fig. 3C and Fig. S3B). Thus, both $CD4^+$ and $CD8^+$ T cells require expression of Myd88, but not TLR4, in order to rapidly respond to innate stimuli.

$CD4^+$ T cell expression of IL-18R and IL-33R is required for maximal innate responses

Given the requirement for Myd88 expression in T cells, we generated mixed BM chimeras to examine various cytokine receptors that utilize this adaptor molecule in downstream signaling (IL-1R, IL-18R, IL-33R, and IFN-γR). Loss of IL-1 or IFN-γ receptor expression had no effect on the innate $CD4^+$ or $CD8^+$ T cell responses to LPS in *Salmonella*-infected mice (Fig. 4A and B, and Fig. S3C and D). In marked contrast, $CD4^+$ or $CD8^+$ T cell expression of IL-18R was essential for rapid amplification of IFN-γ production in response to LPS stimulation (Fig. 4C and Fig. S3E). Interestingly, although IL-33 is usually associated with Th2 cell responses (Lloyd, 2010), $T\text{-}bet^+$ $CD4^+$ T cells lacking the expression of IL-33R demonstrated consistently lower IFN-γ responses to LPS (Fig. 4D). Together, these data indicate that effector $CD4^+$ Th1 cells respond directly to IL-18 and IL-33 in order to maximize IFN-γ production in the presence of innate stimuli.

LPS induction of IL-18 requires inflammasome activity in *Salmonella*-infected mice

Given the requirement for T cell expression of IL-18R and IL-33R, we examined cytokine production in the spleen and liver during innate stimulation with LPS. By RT-QPCR analysis, a modest increase in IFN-γ mRNA was detected in the liver and spleen of uninfected mice that had been injected with LPS (Table 1). However, LPS injection of uninfected mice had little effect on IL-12, IL-18, or IL-33 mRNA in the spleen and only modestly increased these mRNAs in the liver (Table 1). In contrast, injection of LPS into *Salmonella*-infected mice (day 14 post-infection) caused a notable increase in spleen and liver IFN-γ, IL-12, IL-18, and IL-33 mRNA, with the IL-33 increase particularly prominent in the liver (Table 1). At the protein level, injection of LPS into *Salmonella*-infected mice caused a rapid 300-fold increase in IFN-γ and a 35-fold increase in IL-18 in serum (Fig. 5A and B). In contrast, IL-12 and IL-33 concentrations were below the level of detection in the sera of *Salmonella*-infected mice, even after injection with LPS (data not shown), suggesting that the production of these cytokines is restricted to infected tissue, or subject to additional post-transcriptional controls. Injection of flagellin or Pam3CSK4 into *Salmonella*-infected mice also caused a marked increase in serum IFN-γ and IL-18, while injection of Imiquimod did not (Fig. 5C and D), supporting the observation in Figure 1 that T cells can respond to LPS, flagellin or Pam3CSK4 but not Imiquimod stimulation.

A requirement for nucleotide binding domain and leucine rich repeat family (NLR)C4 in flagellin-mediated non-cognate stimulation of $CD8^+$ T cells has previously been reported (Kupz et al., 2012), suggesting that direct recognition of injected flagellin by inflammasome components occurs. Since NLRC4 and NLRP3 play redundant roles in the recognition of *Salmonella* (Broz et al., 2010), we examined whether NLRC4 and NLRP3 were required for non-cognate stimulation of $CD4^+$ T cells by LPS or flagellin. While the injection of wild-type mice with flagellin or LPS induced a rapid increase in serum IL-18, this same response was markedly lower in mice deficient in both NLRC4 and NLRP3 (Fig. 5E). This reduced IL-18 production in response to flagellin or LPS correlated with a severely curtailed innate $CD4^+$ T cell response to either stimuli in mice lacking NLRC4 and NLRP3 (Fig. 5F). A similar requirement for NLRC4 and NLRP3 in the induction of IFN-γ by $CD8^+$ T cells was also observed (Fig. S4). A requirement for NLRC4 and NLRP3 in the innate response of $CD4^+$ T cells to LPS suggested that inflammasome stimulation by live bacteria is required for maximal $CD4^+$ T cell responses to occur. However, $CD4^+$ and $CD8^+$ T cells in mice infected with flagellin-deficient *Salmonella* responded normally to innate stimulation by Ultrapure LPS (Fig 5G and Fig. S4B), indicating that bacterial ligands other than flagellin can also drive the inflammasome activation required for innate T cell stimulation. Together, these data indicate that rapid production of IL-18 in vivo is a critical regulatory checkpoint for non-cognate $CD4^+$ and $CD8^+$ T cell responses to inflammatory stimuli and that this process requires TLR recognition of bacterial ligands and NLR activation in infected mice.

Non-cognate stimulation of T cells contributes to bacterial clearance

In *Salmonella*-infected mice, depletion of $CD4^+$ T cells severely limited bacterial clearance from the spleen and liver (Fig. 6A and B), confirming the importance of $CD4^+$ T cells to host protective immunity (Nauciel, 1990). Depletion of $CD8^+$ T cells also hindered bacterial clearance (Fig. 6A and B), consistent with a secondary role for $CD8^+$ T cells in host protection (Lee et al., 2012a; Nauciel, 1990). Although both $CD4^+$ and $CD8^+$ T cells participate in *Salmonella* clearance, the contribution of cognate versus non-cognate stimulation is currently unknown. In order to assess the in vivo relevance of non-cognate T cell activation, we examined the course of *Salmonella* infection in loxP-*Myd88* mice expressing Cre-recombinase driven by the *Lck* promoter ($Myd88^{fl/fl}$ *Lck-cre*) Early clearance of *Salmonella* was unaffected in $Myd88^{fl/fl}$ *Lck-cre* mice (data not shown), consistent with the dependence of early control on the innate immune compartment (Griffin and McSorley, 2011). However, beginning around 3 weeks post-infection, approximately 1 in 6 $Myd88^{fl/fl}$ *Lck-cre* mice succumbed to primary *Salmonella* infection (Fig. 6C). Furthermore, at 5 weeks post-infection, the spleens of remaining $Myd88^{fl/fl}$ *Lck-cre* mice displayed significantly higher bacterial loads when compared with wild-type and heterozygous littermate controls (Fig. 6D and E). Thus, the ability of T cells to respond to Myd88-dependent signals is an important component in resolving infection with an intra-macrophage pathogen.

Discussion

Approximately 50 years ago, George MacKaness reported that *Brucella*-infected mice display transient cross-reactive protection against other intra-macrophage pathogens and that this effect correlates with the induction of a cellular immune response (Mackaness, 1964). The mechanistic basis of this cross-bacterial protection is usually understood to derive from the indiscriminate killing activity of activated macrophages via reactive oxygen and nitrogen species (Fang, 2004). A lack of target specificity in the efferent phase of host defense against intra-macrophage pathogens is likely to have evolved to combat super-infection or co-infections and is held in check by TCR-mediated clonal expansion and effector development (Jenkins et al., 2001). However, it has become apparent that following clonal expansion, effector T cells can be activated by a variety of non-cognate stimuli (Beadling

and Slifka, 2005; Berg et al., 2002; Freeman et al., 2012; Guo et al., 2012; Kupz et al., 2012; Soudja et al., 2012), suggesting that cognate stimulation may not be required in infected tissues. However, the role of non-cognate T cell stimulation in defense against intra-macrophage pathogens is poorly understood.

Several prior studies have documented the expansion of a large population of activated $CD4^+$ T cells in mice infected with *Salmonella* (Mittrucker et al., 2002; Srinivasan et al., 2004; Srinivasan et al., 2007). Although antigen-specific T cells can be visualized in this infection model using tetramers (Lee et al., 2012b; McSorley et al., 2002; Moon et al., 2007), these populations account for a tiny fraction of the overall polyclonal Th1 cell response to *Salmonella*. Our data show that a large population of T-bet$^+$ $CD4^+$ T cells expands and contracts in concert with tissue bacterial loads and that these $CD4^+$ T cells are required for protective immunity. Furthermore, these expanded Th1 cells transiently gain the ability to secrete IFN-γ when the host is confronted with innate stimuli such as bacterial LPS, flagellin, or Pam3CSK4. The fact that Imiquimod and CpG fail to induce effector responses in this model may mean that cell surface TLRs are more efficient at amplifying the Th1 cell response, however, it is also possible that endosomal TLRs can mediate the same effect if these ligands are internalized, as may occur during infection. Indeed, given the data above, it seems likely that numerous TLR ligands will be capable of amplifying $CD4^+$ T cell responses and that the individual microbe-associated molecular patterns (MAMPs) involved will vary depending on the infection model. The ability of LPS to stimulate $CD4^+$ T cells during *Chlamydia* infection also supports the concept that non-cognate T cell stimulation is common to intra-macrophage infection models.

An important feature of this $CD4^+$ response to innate stimuli is that it is maximally effective during the period of active infection. Therefore, during active *Salmonella* infection, effector $CD4^+$ Th1 cells have a reduced threshold for stimulation and IFN-γ production can be elicited immediately upon encountering an appropriate cytokine milieu in inflamed tissues. The ability to induce effector functions without a requirement for recognizing cognate antigen and MHC on an infected cell has probably evolved to enhance the efficiency of the adaptive response to infection. This may well be a common feature of the T cell response to many microbial pathogens. Surprisingly, the heightened response of $CD4^+$ T cells to LPS-induced inflammation during infection required the expression of NLRC4 and NLRP3. The most likely explanation for this finding is that inflammasome sensing of intracellular bacterial components synergizes with TLR recognition of MAMPs to induce cytokine production. Indeed, the MAMPs that efficiently induce IFN-γ production from $CD4^+$ and $CD8^+$ T cells are also able to rapidly increase concentrations of circulating IL-18. This model of T cell stimulation is distinct from a previous study (Kupz et al., 2012), which suggested that injected flagellin has to be transported into dendritic cells for cytosolic recognition by NLRC4, thus inducing non-cognate stimulation of memory $CD8^+$ T cells. Indeed, our data demonstrate that flagellin expression by *Salmonella* is not essential for non-cognate T cell stimulation to occur, although this finding may also reflect other differences between these two models.

Having a reduced threshold for effector T cell stimulation is likely to provide an important evolutionary advantage when combating rapidly dividing pathogens such as *Salmonella*. It also suggests that activated $CD4^+$ T cells may provide cross-reactive immunity against co-infections, although we have not yet examined this possibility directly. While our model required the injection of MAMPs to uncover the mechanism of non-cognate T cell stimulation, this same pathway likely contributes to the endogenous production of IFN-γ in infected tissues. Indeed, the fact that *Myd88*$^{fl/fl}$ *Lck-cre* mice have difficulty resolving primary infection with *Salmonella* supports an important role for this non-cognate pathway in primary clearance. Further studies will be required to determine whether non-cognate

stimulation can provide protection during co-infections, or conversely, whether this same innate inflammatory mechanism plays a role in immune-mediated pathology (Raetz et al., 2013).

Various inflammatory cytokines and TLR ligands are known to activate effector T cells in vitro and in vivo (Beadling and Slifka, 2005; Berg et al., 2002; Freeman et al., 2012; Guo et al., 2012; Kupz et al., 2012; Soudja et al., 2012). Indeed, a role for MAMP-induced IL-18 has previously been described in the stimulation of $CD8^+$ T cells and NK cells in mouse models of *Listeria* infection, LPS toxicity, and inflammation-induced T cell turnover (Lertmemongkolchai et al., 2001; Pien et al., 2002; Tough et al., 2001). For Th1 cells, IL-12 and IL-18 can stimulate IFN-γ production in vivo (Robinson et al., 1997; Yoshimoto et al., 1998), while IL-33R stimulation in Th2 cells can induce IL-13 (Guo et al., 2009). However, the contribution of these non-cognate T cell responses to the resolution of infection in vivo is unclear. Our BM chimera data show that $CD4^+$ T cell expression of Myd88, IL-18R, and IL-33R each impact maximal IFN-γ production, but that intrinsic IL-1R or TLR4 expression is not required. Thus, $CD4^+$ Th1 cells likely respond to a mix of IL-18 and IL-33 in infected tissue and mRNA for both of these cytokines was increased in the liver and spleen after LPS injection. Although IL-33R makes a modest contribution to the splenic Th1 cell response, this may be because at high concentrations of IL-18R signaling this pathway becomes largely redundant, thus masking the pro-inflammatory role of IL-33. Future experiments will determine whether IL-33 can play a more prominent role in response to other TLR ligands or under physiological conditions.

Together, our data provide a mechanistic framework for understanding non-cognate stimulation of $CD4^+$ and $CD8^+$ T cells and the contribution of this response in defense against intra-macrophage pathogens. Greater understanding of this pathway may lead to the development of effective immunomodulatory therapeutics for the treatment of persistent infection, as well as highlighting potential mediators of immunopathology for more targeted immunosuppressive interventions.

Experimental procedures

Mice

C57BL/6, B6.SJL-$Ptprc^aPepc^b$/BoyJ, B6.PL-$Thy1^a$/CyJ, TLR4-deficient, and IL-18R-deficient mice were purchased from The Jackson Laboratory or NCI at 6–8 weeks of age. MyD88-, IL-1R1-, and IFN-γR-deficient C57BL/6 mice were obtained from Dr. Jenkins (University of Minnesota) and Dr. Way (University of Cincinnati), and bred in our animal facility. IL-33R-deficient C57BL/6 mice were kindly provided by Dr. Bryce (Northwestern University). Mice deficient in NLRC4 and NLRP3 were maintained at Stanford University (Broz et al., 2010). T cell specific Myd88 deficient mice were generated by crossing *Lck-cre* mice to *Myd88*$^{flox/flox}$ flanked mice purchased from Jackson labs. Mice were genotyped by PCR according to protocols provided by The Jackson Laboratory. All animal procedures were approved by UC Davis IACUC (#16612).

Bacterial strains and infection

Salmonella strains utilized in this study are listed in Supplemental Table 1. C57BL/6 mice were infected iv with 5×10^5 BRD509 Δ*aroAD* strain of *Salmonella enterica* serovar Typhimurium or an aflagellate BRD509 mutant SPN529 that was constructed as described in Supplemental data. *Salmonella* were grown overnight in static LB broth cultures at 37°C before being washed and resuspended in PBS. Bacterial concentration was estimated by spectrophotometry at OD600 to prepare the challenge inoculum. The actual dose administered to mice was determined by serial dilution and plating on MacConkey agar

plates. *Chlamydia muridarum* strain Nigg II was purchased from ATCC (Manassas, VA) and cultured in HeLa 229 cells in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Elementary bodies (EBs) were purified by discontinuous density gradient centrifugation as previously described and stored at −80°C (Scidmore, 2005). The number of inclusion-forming units (IFUs) of purified EBs was determined by infection of HeLa 229 cells and enumeration of inclusions stained with anti-*Chlamydia* MOMP. A fresh aliquot was thawed and used for every infection experiment. C57BL/6 mice were injected iv with 1×10^7 *C. muridarum*.

Determination of bacterial loads

Mice were euthanized and the indicated organs collected in HBSS (Hank's balanced salt solution) or PBS (phosphate buffered saline, both Gibco) on ice, homogenized, and reconstituted in a known volume of HBSS. Samples were mixed thoroughly and serial 1:10 dilutions were plated on MacConkey agar plates (Becton Dickinson), incubated overnight at 37°C, and enumerated the following day to calculate the number of colony forming units (cfu) in the total organ.

Bone marrow chimeras

Mixed bone marrow chimeras were generated by first irradiating 45.1+ congenic mice (B6.SJL) (1000 rads irradiation by cesium source). The following day, bone marrow was isolated from congenic CD90.1+ (B6.PL) and genetically-deficient mouse femurs and tibias, cells counted by hematocytometer, and combined at a 1:1 ratio before being administered (4×10^6 total cells) in 200μl 1X PBS. After BM transfer, chimeras were maintained on antibiotics for at least 4 weeks and blood collected 4 weeks later for flow cytometric analysis of immune reconstitution. Chimeras were infected 6–8 weeks after administration of bone marrow, and at least 2 weeks after discontinuation of antibiotic treatment.

In vivo stimulation with TLR ligands

Ultrapure lipopolysaccharide (LPS) from *E. coli* strain EH100Rα (Alexis, TLRgradeR), purified bacterial flagellin, endotoxin-free Pam3CSK4, CpG DNA (ODN1585), and Imiquimod (Invivogen) were diluted in 1X PBS (UltraPure Phosphate Buffered Saline, Gibco) and administered IV to mice. Spleens and livers were harvested from infected or uninfected mice 4 hours or less after administration of MAMPs. Flagellin was purified from an LPS-deficient X4700 strain of *S.* Typhimurium using a modified acid-shock protocol and passed multiple times through Detoxi-Gel columns (Thermo Scientific) to remove residual endotoxin, as previously described (Salazar-Gonzalez et al., 2007).

Flow cytometry

For flow cytometry, cells were prepared as a single cell suspension of between 1×10^6 and 8×10^6 cells/ml and stained with various antibodies from eBioscience, Becton Dickinson (BD), or Tonbo Biosciences, for 30 minutes to 1 hour in Fc block on ice. Intracellular cytokine or transcription factor staining was performed using the Foxp3 intra-nuclear staining kit from eBioscience, as recommended by the manufacturer. Stained cells were analyzed using a FACS Canto, FACS Aria or Fortessa (BD) using appropriate compensation controls and flow cytometry data was analyzed using FlowJo software (TreeStar).

RNA preparation and RT-QPCR

RNA was extracted from cryopreserved spleens and livers according to the manufacturer's instructions using TRIzol reagent (Ambion) and quantified using a Nanodrop spectrophotometer (Thermo Scientific). RNA was DNase-treated using a DNA-free kit

(Ambion), and a SensiFAST SYBR Hi-ROX One-Step RT-QPCR kit (Bioline) was used for both cDNA synthesis and QPCR reactions using 100ng total RNA per reaction. RT-QPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in a 96 well plate and triplicate data was analyzed in Microsoft Excel by the comparative Ct method (Applied Biosystems) using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the control. Data is expressed as fold change of stimulated over unstimulated ΔCt. Primer sequences and references are listed in Supplementary Table 2.

Serum cytokine ELISAs

Sera was prepared by collecting blood from retro-orbitally exsanguinated, anesthetized mice that had been infected or stimulated as indicated, blood incubated on ice to allow clotting, then centrifuged, serum harvested and stored at −20°C. Cytokine ELISAs for IFN-γ, IL-12 and IL-33 were performed according to the instructions provided by the Ready-Set-Go kit (eBioscience) and concentrations determined using the protein standard provided. For the IL-18 ELISA, the same standard protocol was followed using capture and detection antibodies (Medical and Biological Labs, Co) as recommended by the manufacturer with rmIL-18 as a standard (R&D). After substrate was added, plates were read at 450nm using a microplate reader (Spectra Max M2, Molecular Devices, Inc) and analyzed in Microsoft Excel.

Antibody depletion of $CD4^+$ and $CD8^+$ T cells

Loss of $CD4^+$, $CD8^+$, or $CD4^+$ and $CD8^+$ T cells was examined in vivo by depletion of cells with indicated rat anti-mouse monoclonal antibodies (BioXCell). Antibodies were administered intraperitoneally twice per week starting at day 7 and depletions were maintained until euthanization. The initial doses (days 7 and 10) contained 200 μg of antibody per mouse, all subsequent doses contained 300 μg per mouse. Maintenance of depletion was monitored by flow cytometry on blood collected once per week from the lateral tail vein.

Statistical Analyses

All statistical analyses were performed as described in the figure legends using Prism version 5 (Graphpad). All error bars are displayed as mean +/− SEM (standard error mean). $P< 0.005$ (***), $p< 0.01$ (**), $p<0.05$ (*) or $p>0.05$ (ns).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- $CD4^+$ T cell effector functions are elicited by MAMPs during bacterial infection
- T cell-intrinsic Myd88, IL-18R, and IL-33R maximize stimulation of $CD4^+$ Th1 cells
- TLR and inflammasome components amplify $CD4^+$ T cell responses
- Loss of innate T cell stimulation impairs host resolution of bacterial infection

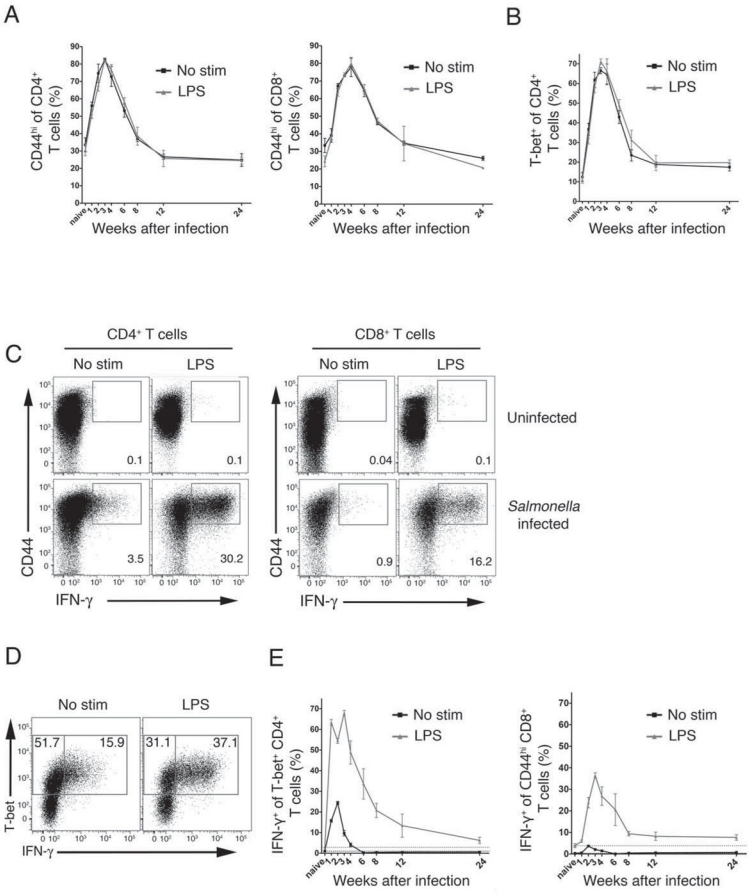

Figure 1. Expanded CD4$^+$ Th1 and CD8$^+$ T cells acquire the ability to respond to innate stimulation

C57BL/6 mice were infected IV with 5×10^5 *Salmonella* (BRD509) and at various times later, the proportion of CD4$^+$ and CD8$^+$ T cells in the spleen that express CD44 and IFN-γ or T-bet on CD4$^+$ T cells was assessed by flow cytometry. Four hours prior to analysis, mice were injected IV with 10μg LPS (LPS) or received no additional stimulus (No stim). (A, B, E) Graphs show change in the percentage of (A) CD44$^+$, (B) Tbet$^+$, or (E) IFN-γ$^+$ and Tbet$^+$ (percentage of Th1 cell producing IFN-γ) for CD4$^+$ T cells and IFN-γ$^+$ and CD44hi (percentage of activated CD8$^+$ T cells producing IFN-γ) for CD8$^+$ T cells in the spleen. (C, D) Representative flow cytometry plots showing the production of IFN-γ at day 14 after infection by (C) CD44$^+$ CD4$^+$ and CD8$^+$ T cells, or (D) T-bet$^+$ CD4$^+$ T cells. Numbers show the percentage of IFN-γ positive or negative T cells within the boxed gates. Experiments contained at least 3 mice/group at each time point and representative flow cytometry plots show data that are representative of at least three experiments. Error bars represent mean +/− SEM. See also Figure S1.

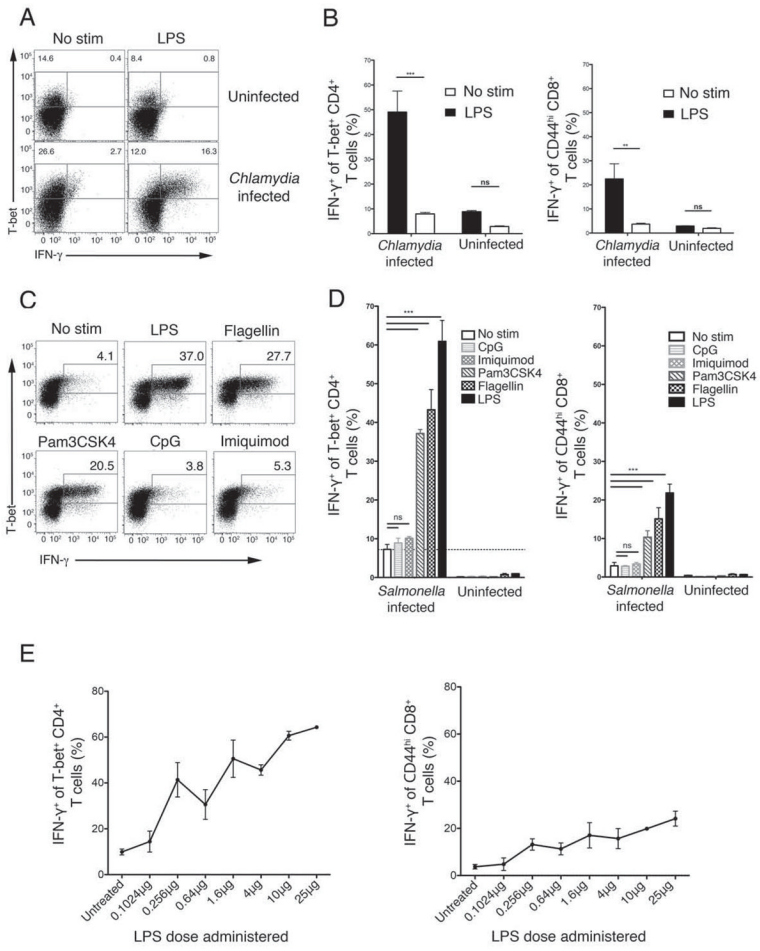

Figure 2. Amplification of CD4$^+$ Th1 and CD8$^+$ T cell responses occurs with multiple innate ligands and in multiple intra-macrophage infections

C57BL/6 mice were infected IV with 1×10^7 *Chlamydia muridarum* EBs or 5×10^5 *Salmonella*. One (*Chlamydia*) or two (*Salmonella*) weeks later, infected or uninfected mice were injected IV with LPS, flagellin, CpG DNA, Imiquimod, or Pam3CSK4 and spleens harvested four hours later to determine IFN-γ production. (A–B) Representative flow cytometry plots and bar graph of combined data showing CD44 or intracellular T-bet and IFN-γ staining in *Chlamydia*-infected mice after gating on CD4$^+$ or CD8$^+$ as indicated. (C–D) Representative flow cytometry plots and bar graph of combined data showing CD44 or intracellular T-bet and IFN-γ staining in *Salmonella*-infected mice after gating on CD4$^+$ or CD8$^+$. (E) Graph showing percentage of IFN-γ$^+$ cells after IV LPS administration at various doses. CD4$^+$ are shown as percentage of total Tbet$^+$ cells producing IFN-γ and CD8$^+$ as percentage IFN-γ$^+$ of total CD44^{hi}. (B,D) Statistical significance was determined by two-way ANOVA with a bonferroni post-test. P< 0.005 (***), p<0.01 (**), or p>0.05 (ns). All experiments contain at least 3 mice/ group and were conducted at least twice. Error bars represent mean +/− SEM. See also Table S1.

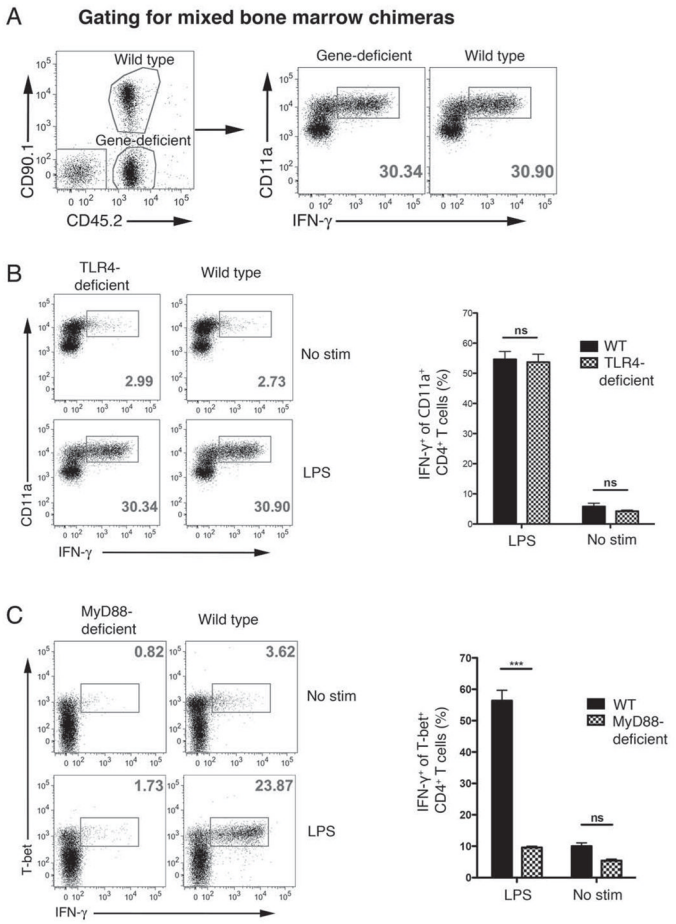

Figure 3. Innate Th1 cell stimulation by LPS requires T cell intrinsic expression of MyD88

$CD45.1^+$ C57BL/6 mice were irradiated and reconstituted with a mixture of bone marrow from wild-type ($CD90.1^+$ $CD45.2^+$) and TLR4-, or Myd88-deficient ($CD90.2^+$ $CD45.2^+$) mice. After immune reconstitution BM chimeras were infected IV with 5×10^5 *Salmonella* and response to LPS determined in the spleen. (A) Gating strategy used to examine donor wild-type and gene-deficient $CD4^+$ T cells in mixed bone marrow chimeras. Splenocytes were gated as live, singlets, and $CD4^+$ $CD8^-$, then gated as shown in the left panel for congenic markers. Each gated cell population was assessed for IFN-γ production and either CD11a (TLR4) or T-bet (Myd88). (B–C) Representative flow cytometry plots and a graph of combined data are shown for (B) TLR4-deficient, and (C) MyD88-deficient chimeras. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/− SEM. See also Figure S2.

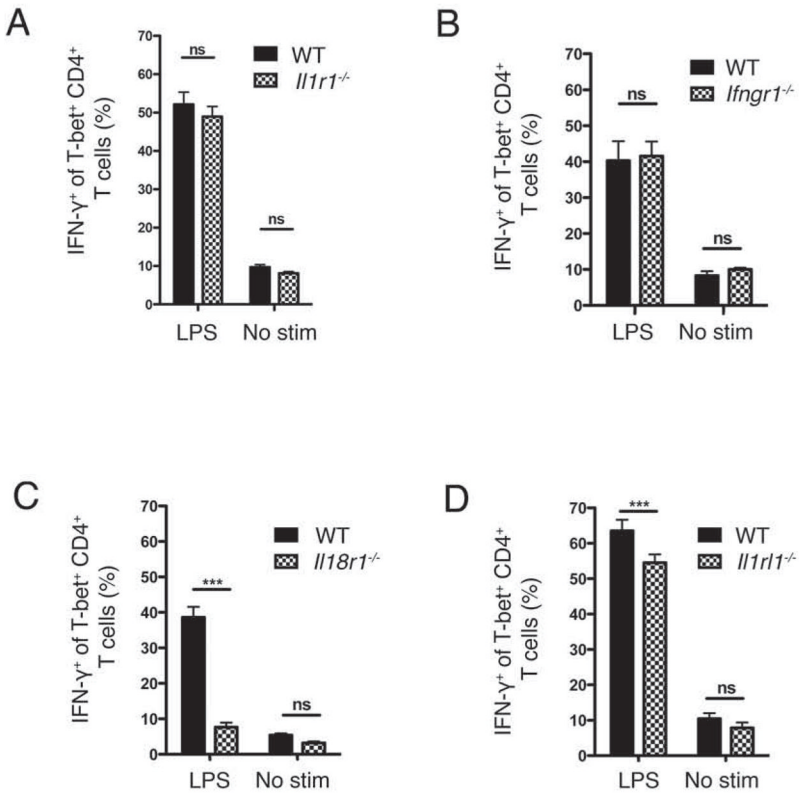

Figure 4. Maximal Th1 cell stimulation by LPS requires T cell intrinsic expression of IL-18R and IL-33R

Mixed BM chimeras were generated, infected, and stimulated, as described in Figure 3. A graph showing the percentage of IFN-γ^+ Th1 cells in wild-type versus gene-deficient $CD4^+$ T cells is shown for (A) Il1r1, (B) Ifngr1, (C) Il18r1, and (D) Il1rl1 (IL33R) mixed BM chimeras. All experiments include at least 3 mice per group and were performed at least twice with similar results. Statistical analyses were performed by two-way repeated measures ANOVA with bonferroni post-test. Error bars represent mean +/− SEM. See also Figure S3.

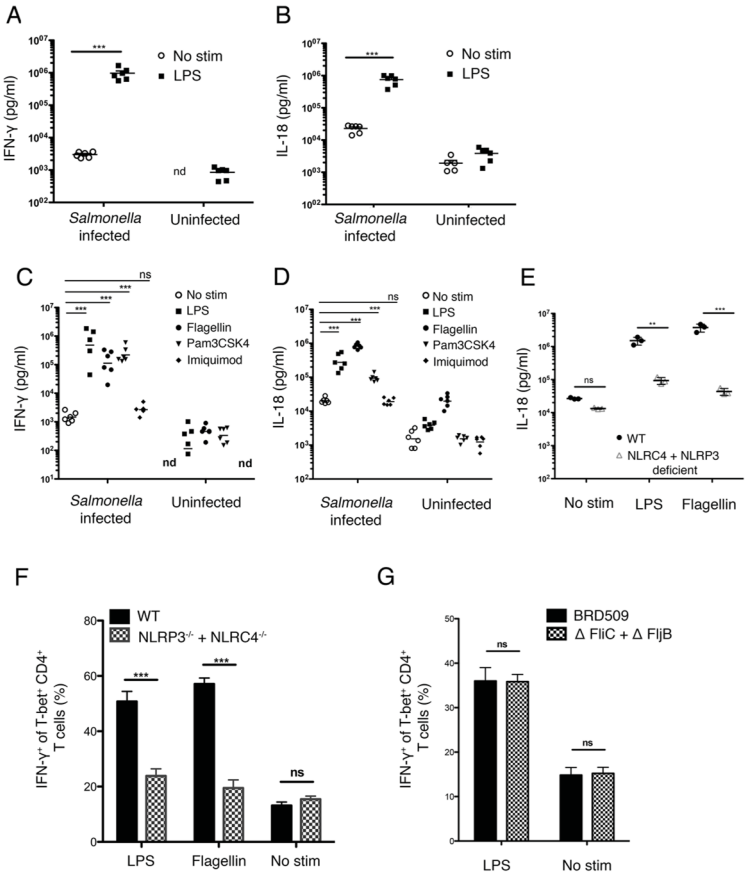

Figure 5. Production of IL-18 and amplification of CD4$^+$ Th1 cell responses requires inflammasome components

Wild-type or mice deficient in NLRP3 and NLRC4 were infected IV with 5×10^5 *Salmonella* (BRD509 or flagellin-deficient BRD509) and the splenic response to LPS, flagellin, Pam3CSK4, and Imiquimod injection determined 2 weeks later, and four hours after stimulation. (A–E) Plots show serum cytokine concentrations as determined by ELISA, for (A, C) IFN-γ and (B, D, E) IL-18. Data shown are pooled from 2 experiments. (F) IFN-γ production by Th1 cells in *Salmonella*-infected wild-type, or mice lacking NLRP3 and NLRC4, after administration of LPS or flagellin. Data is pooled from 2 experiments each with more than 3 mice per group. (G) IFN-γ production by Th1 cells after LPS stimulation of mice infected with BRD509 (flagellin-expressing) or a flagellin deficient mutant of BRD509 (ΔfliCΔfljB). Data is representative of at least 2 experiments with at least 3 mice per group. Statistical significance was determined by two-way ANOVA with a bonferroni post-test. Error bars represent mean +/− SEM. See also Figure S4.

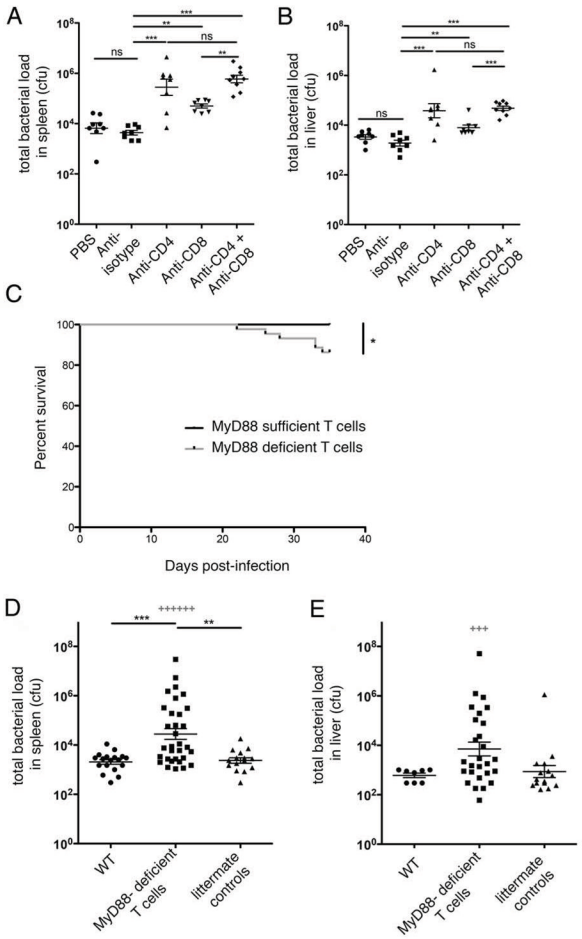

Figure 6. Mice lacking T-cell expression of Myd88 are more susceptible to *Salmonella* infection

(A–B) T cell-depleted, or (C–E) Wild-type, *Lck-cre x Myd88*$^{flox/flox}$ homozygote and *Lck-cre x Myd88*$^{flox/WT}$ heterozygote mice were infected IV with 5×10^5 *Salmonella.* (A–B) Mice given PBS, anti-CD4, anti-CD8, both, or an isotype control antibody were sacrificed at day 30 post-infection and bacterial loads in the spleen and liver were determined. For *Lck-cre x Myd88*$^{flox/flox}$ and *Lck-cre x Myd88*$^{flox/WT}$ mice (C) survival or (D–E) bacterial burdens in the spleen and liver were monitored five weeks after infection. (C) Survival curve after *Salmonella* infection. Graph shows combined data from at least 3 experiments and contains at least 10 mice per group. Wild-type and littermate control *Lck-cre x Myd88*$^{flox/WT}$ mice are grouped together. Statistical significance was observed by the Log-rank (Mantel-Cox) test. (D–E) Bacterial loads in the (D) spleen and (E) liver of individual mice, 5 weeks post-infection with BRD509 in *Lck-cre x Myd88*$^{flox/flox}$ and *Lck-cre x Myd88*$^{flox/WT}$ and wild type mice. Gray (+) symbols show the number of mice that became moribund prior to assessment of bacterial burdens at the 5 week time point. Statistical significance was determined on log transformed data by one-way ANOVA. Error bars represent mean +/− SEM

Table 1

LPS stimulation leads to increased mRNA expression of IFN-γ, IL-12, IL-18, and IL-33

C57BL/6 mice were infected IV with 5×10^5 *Salmonella* and two weeks later, infected or uninfected mice were injected IV with LPS. Spleens and livers were harvested four hours later and RT-QPCR performed on extracted mRNA. Numbers shown are normalized to gapdh expression and indicate the fold-change increase over unstimulated mice. See also Table S2.

		effect of LPS (relative to unstimulated controls)			
Spleen	**gene:**	**IFN-γ**	**IL-12**	**IL-18**	**IL-33**
	uninfected	1	1	1	1
	uninfected + LPS	8.67	0.99	1.30	1.60
	Salmonella-infected	1	1	1	1
	Salmonella-infected+ LPS	22.02	4.52	2.26	5.35
Liver					
	uninfected	1	1	1	1
	uninfected + LPS	11.07	1.47	1.96	6.16
	Salmonella-infected	1	1	1	1
	Salmonella-infected+ LPS	27.9	5.36	2.6	16.2