The Journal of Infectious Diseases
MAJOR ARTICLE

Role of Interleukin 36γ in Host Defense Against Tuberculosis

Fadhil Ahsan,[1,2,a] Pedro Moura-Alves,[1,a] Ute Guhlich-Bornhof,[1] Marion Klemm,[1] Stefan H. E. Kaufmann,[1] and Jeroen Maertzdorf[1]

[1]Department of Immunology, Max Planck Institute for Infection Biology, and [2]ZIBI Graduate School Berlin, Germany

Tuberculosis remains a major killer worldwide, not the least because of our incomplete knowledge of protective and pathogenic immune mechanism. The roles of the interleukin 1 (IL-1) and interleukin 18 pathways in host defense are well established, as are their regulation through the inflammasome complex. In contrast, the regulation of interleukin 36γ (IL-36γ), a recently described member of the IL-1 family, and its immunological relevance in host defense remain largely unknown. Here we show that *Mycobacterium tuberculosis* infection of macrophages induces IL-36γ production in a 2-stage-regulated fashion. In the first stage, microbial ligands trigger host Toll-like receptor and MyD88-dependent pathways, leading to IL-36γ secretion. In the second stage, endogenous IL-1β and interleukin 18 further amplify IL-36γ synthesis. The relevance of this cytokine in the control of *M. tuberculosis* is demonstrated by IL-36γ–induced antimicrobial peptides and IL-36 receptor–dependent restriction of *M. tuberculosis* growth. Thus, we provide first insight into the induction and regulation of the proinflammatory cytokine IL-36γ during tuberculosis.

Keywords. IL-36γ; *Mycobacterium tuberculosis*; TLR; inflammasome; antimicrobial peptide.

Mycobacterium tuberculosis is an intracellular bacterium that causes a profound health burden worldwide [1]. Macrophages serve as preferred habitat and are major effector cells [2, 3]. The outcome of *M. tuberculosis* infection is strongly determined by host innate inflammatory responses, which serve as a first line of defense [2, 3]. After host encounter with *M. tuberculosis,* proinflammatory cytokines rapidly initiate and then sustain defense responses [4]. These molecular events are driven mainly by specific host pattern-recognition receptors (PRRs) upon recognition of pathogen-associated molecular patterns (PAMPs). Myeloid differentiation primary response gene 88 (MyD88), the shared adaptor for most Toll-like receptors (TLRs) and interleukin 1 (IL-1) receptors, is critical for the induction and coordination of host protective mechanisms in tuberculosis [5, 6]. Yet our knowledge of specific regulatory mechanisms involved in innate immune defense remains incomplete [2, 3, 7].

Several studies have established the critical role of IL-1β and interleukin 18 (IL-18) in host resistance to tuberculosis [8, 9], as well as their regulation by cysteine aspartic acid protease (caspase)–inflammasome complexes, which are responsible for their posttranslational processing into active forms [10–12]. Several inflammasome components, such as NOD-like receptor family, pyrin domain containing 3 (NLRP3) and absent in melanoma 2 (AIM2), can be activated by *M. tuberculosis*, leading to the secretion of IL-1β and IL-18 [13–16]. In contrast, the regulation and role of other IL-1 family members, such as interleukin 36 (IL-36) cognates [17, 18], remain poorly understood.

The IL-36 subfamily comprises the proinflammatory molecules IL-36α, IL-36β, and IL-36γ and the putative IL-36 receptor antagonist (IL-36RN) [18]. The signaling through a specific IL-36 receptor (IL-36R) induces type 1 immune responses, which are critical for control of intracellular bacterial pathogens [19, 20]. It is noteworthy that, in contrast to mice, it has been reported that human myeloid cells but not T cells express IL-36R [21]. This raises questions about the role and regulation of IL-36 in human innate immune cells. In host defense against *M. tuberculosis*, macrophages play a central role as the major professional phagocytes, and epithelial cells serve as an alternative niche of *M. tuberculosis* persistence [2, 22, 23]. Macrophages are not only the main cells involved in bacterial clearance, but are also a central habitat for *M. tuberculosis*, where the bacteria prevent phagosomal killing, allowing for a safe niche to reside and replicate [2, 3]. First-line defense during infection is mediated by direct phagosomal killing and release of antimicrobial peptides (APs), as well as effector mechanisms induced by proinflammatory factors [2, 23–25]. Recent studies have shown that IL-36 cognates can induce APs, including cathelicidin and beta defensins, in human keratinocytes [26, 27]. These APs are important effectors of host defense, capable of controlling mycobacterial growth [28, 29]. In human cells, these APs can also be induced upon *M. tuberculosis* infection and by stimulation with vitamin D [24, 25, 28, 29]. Although the function of APs in antibacterial host defense is well established, the

Received 22 December 2015; accepted 7 April 2016; published online 18 April 2016.
[a]F. A. and P. M.-A. are co–first authors.
Correspondence: S. H. E. Kaufmann, Department of Immunology, Max Planck Institute for Infection Biology, Berlin, Germany (kaufmann@mpiib-berlin.mpg.de).

The Journal of Infectious Diseases® 2016;214:464–74

DOI: 10.1093/infdis/jiw152

potential involvement of IL-36 in *M. tuberculosis* control in human cells remains elusive. Therefore, the host-regulatory mechanisms leading to IL-36 production and their relevance for host defense against tuberculosis need to be elucidated.

Here we describe the induction of IL-36γ in macrophages upon *M. tuberculosis* infection and its relevance in *M. tuberculosis* growth restriction. We uncover a dual-stage induction of IL-36γ: an initial stage involving the recognition of *M. tuberculosis* PAMPs via TLRs and MyD88 pathways and a second regulatory stage in which endogenous IL-1β and IL-18 further amplify the production of IL-36γ. Furthermore, we have identified several APs that are induced in an IL-36γ–dependent manner and possibly account for the IL-36–mediated bacterial growth inhibition in macrophages.

METHODS

Human Cell Culture and Macrophage Differentiation

THP-1 human monocytic cell line–derived macrophages (THP-1 macrophages), primary human monocyte-derived macrophages (MDMs), and peripheral blood mononuclear cells (PBMCs) were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Darmstadt, Germany). A549 cells (human type II pneumocytes) and HEK293T (human embryonic kidney epithelial cells) were cultured in Dulbecco's modified Eagle's medium. Both culture media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 1% (v/v) sodium pyruvate (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-glutamine, 1% (v/v) HEPES buffer (Gibco), and 0.05 M 2-mercaptoethanol (Gibco). THP-1 cells (human monocytic cell line) were differentiated into macrophages, using 100 nM phorbol myristate acetate. Generation of small hairpin RNA knockdown THP-1 cells and cell stimulation are provided in Supplementary Methods and Table 1. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. For the generation of human macrophages, peripheral blood $CD14^+$ cells were positively selected by magnetic-activated cell sorting using the $CD14^+$ cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's instructions, and differentiated with granulocyte macrophage colony-stimulating factor at 20 ng/mL for 7 days in Roswell Park Memorial Institute 1640 medium. Cells were kept at 37°C in 5% CO_2. Buffy coats were obtained from the German Red Cross (Deutsches Rotes Kreuz; ethical registration no. EA1/353/14).

Generation of Murine Bone Marrow–Derived Macrophages

Bone marrow–derived macrophages (BMDMs) were obtained from femoral and tibial bones of 8–14-week-old C57BL/6 mice and maintained in Dulbecco's modified Eagle's medium containing 20% L929 cell supernatant as a source of granulocyte macrophage colony-stimulating factor, 10% (v/v) heat-inactivated fetal bovine serum, 5% heat-inactivated horse serum, 1% (v/v) L-glutamine, and 1% (v/v) sodium pyruvate. *Myd88*$^{-/-}$ knockout mice were kindly provided by Drs Kiyoshi Takeda and Shizuo Akira, Osaka University (Osaka, Japan). *Il1b*$^{-/-}$ and *Il18*$^{-/-}$ mice were kind gifts of Dr Bärbel Raupach [30] and originally provided by D. Chaplin [31] and K. Takeda [32], respectively. *Casp1*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice [33] were kindly provided by Genentech (San Fransisco, California).

Mycobacterial Culture Condition

M. tuberculosis strain H37Rv was grown in Middlebrook 7H9 broth (BD, Heidelberg, Germany), supplemented with 0.05% glycerol, Tween 80, and 10% ADC enrichment (BD), to an early log phase.

Preparation of *M. tuberculosis* for In Vitro Infection

Bacteria were maintained in log growth phase at an OD_{600} of 0.2–0.6. Before cell infection, bacteria were centrifuged and resuspended with phosphate-buffered saline. Single bacteria were obtained using the syringe method and resuspended in culture medium at desired concentrations. An OD of 1 is equivalent to 2×10^8 bacteria. All infections were done at a multiplicity of infection of 10 unless stated otherwise.

RNA Extraction and Quantitative Polymerase Chain Reaction (qPCR) Analysis

RNA derived from human and murine samples was isolated using Trizol (Life Technologies, Ober-Olm, Germany), in experiments with *M. tuberculosis* infection. In experiments without *M. tuberculosis* infection, RNAeasy Plus kit (Qiagen, Hilden, Germany) was used for RNA extraction. After quantification of the RNA concentration with Nanodrop (Nanodrop 2000c, Thermo Scientific, Darmstadt, Germany), RNA underwent reverse transcription at an equal concentration, using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany), and then was subjected to real-time qPCR analysis, using Power SYBR Green (Applied Biosystems, Darmstadt, Germany) in a LightCycler480 thermocycler (Roche, Berlin, Germany). Primers are listed in Supplementary Table 2. *B2M* was used as an internal control. All gene expression data were normalized to the average values for uninfected/small hairpin RNA scramble control samples or dimethyl sulfoxide–stimulated samples. Fold change was determined using the normalized cycle threshold (Ct) value, assuming that a ΔCt of 1 equals a fold change of 2.

Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants were harvested from infected or stimulated cells. Prior to assay, supernatants of infected cells were filtered through a SPIN-X tube (Sigma-Aldrich, Munich, Germany). Samples were assayed using ELISA kits for IL-36γ (Cusabo Biotech, Wuhan, China), IL-18 (MBL, Leuven, Belgium) and IL-1β and IL-6 (both R&D Sytems, Minneapolis, Minnesota). All procedures were performed according to manufacturer's instructions.

Immunoblotting

Cells were lysed in 1× RIPA buffer supplemented with a cocktail of protease inhibitors (Santa Cruz Biotechnology, Heidelberg, Germany) and centrifuged for 20 minutes in filter SPIN-X

tubes. Soluble proteins were diluted at equal concentrations in 2× Laemmli buffer and denatured at 95°C for 5 minutes. Cell lysates were separated by 4%–15% Mini Protean sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad) and transferred onto a nitrocellulose membrane (Roche). Blots were incubated with antibodies specified in the Supplementary Materials. Beta actin was used as loading control with a 42-kD band.

Bacterial Growth Quantification

Colony-forming units (CFUs) and uracil uptake were measured in parallel for bacterial growth. For CFUs, cells were lysed by 0.1% Triton-X in dH20 and serially diluted. Bacteria were plated onto 7H11 Middlebrook agar, sealed with paraffin film, and incubated for 21 days at 39°C.

Statistical Analysis

GraphPad Prism, version 5, was used for statistical analyses. The Student *t* test was used for single comparisons, and 1-way analysis of variance was performed for multiple comparisons, followed by the Bonferroni post hoc test. Data derived from PBMCs and MDMs were analyzed using the nonparametric Mann–Whitney *t* test. All data are representative of at least 3 biological replicates of each independent experiment and are depicted as mean ± SD, unless otherwise noted.

RESULTS

IL-36γ Expression Upon *M. tuberculosis* Infection

We first interrogated whether *M. tuberculosis* infection induces IL-36 expression. Various human and murine cell types were infected with *M. tuberculosis*, and expression of genes encoding IL-36α, IL-36β, IL-36γ, and IL-36RN was evaluated at various time points after infection. With the exception of A549 cells, gene expression of all IL-36 cognates was induced in all cell types tested (ie, PBMCs, MDMs, THP-1 macrophages, and

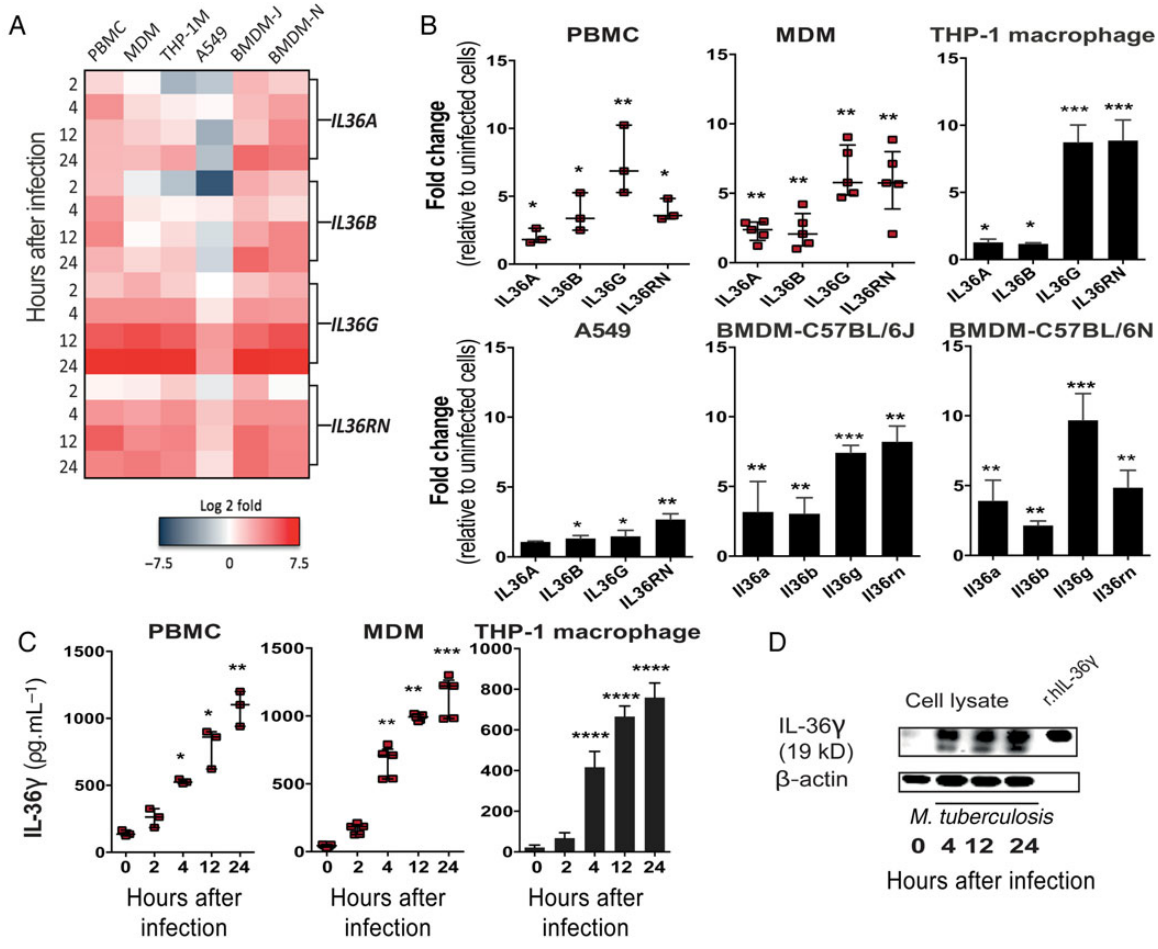

Figure 1. Interleukin 36γ (IL-36γ) induction upon *Mycobacterium tuberculosis* infection. *A*, Heat map of gene expression of IL-36 cognates (*IL36A*, *IL36B*, *IL36G*, and *IL36RN*) in different cells upon *M. tuberculosis* infection 2, 4, 12, and 24 hours after infection. Expression relative to uninfected cells. Upregulation and downregulation shown in red and dark blue, respectively. *B*, Gene expression of IL-36 cognates 4 hours after infection, relative to uninfected controls. *C*, IL-36γ protein levels in supernatants of infected cells. *D*, Immunoblot of intracellular IL-36γ from *M. tuberculosis*–infected THP-1 cell lysates. Recombinant human IL-36γ (r.hIL-36γ) was used as a control. All data are representative of 4 independent experiments, except for peripheral blood mononuclear cell (PBMC) and monocyte-derived macrophage (MDM) data, which represent 3 independent experiments. Data for PBMCs and MDMs are depicted as medians (interquartile ranges), with each dot representing an individual donor. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, and $^{****}P \leq .0001$. Abbreviation: THP-1 M, THP-1 macrophages.

murine BMDMs from C57BL/6 strains J [C57BL/6J] and N [C57BL/6N]; Figure 1*A*). Induction of IL-36γ was dependent on time and multiplicity of infection (Figure 1*A* and Supplementary Figure 1*A*–*F*), showing an upregulation in gene expression as early as 4 hours after infection (Figure 1*B*). Consistently, a similar pattern of IL-36γ protein release (Figure 1*C*) and expression in cell lysates of THP-1 macrophages (Figure 1*D*) was observed.

MyD88-TLR2/4–Dependent IL-36γ Production Upon *M. tuberculosis* Infection

Next we determined whether IL-36γ production in response to *M. tuberculosis* infection is MyD88 dependent. To this end, we generated *MYD88* KD macrophages, using small hairpin RNA (Supplementary Figure 2*A*). Upon *M. tuberculosis* infection, gene expression and secretion of IL-36γ was significantly impaired in *MYD88* KD THP-1 macrophages, as compared to scramble control cells (Figure 2*A*). Coherently, we found that BMDMs from *Myd88* $^{-/-}$ mice failed to induce upregulation of IL-36γ messenger RNA (mRNA; Figure 2*B*). No difference in cell viability was detected between KD and scramble control cells (Supplementary Figure 2*B*). These results suggest an involvement of MyD88-related TLRs in IL-36γ production. To identify these, we analyzed several TLR agonists for their capacity to induce IL-36γ expression [34]. TLR2 ligands, including Pam3CSK4, HKLM, and FSL1, as well as the TLR4 ligand lipopolysaccharide (derived from *Escherichia coli*), rapidly induced IL-36γ mRNA (Supplementary Figure 2*C*). It is noteworthy that induction of IL-36γ was independent from the presence of endotoxin in the ligands tested (Supplementary Figure 2*D*). To assess the TLR2 and TLR4 dependency of IL-36γ induction by *M. tuberculosis*, we generated *TLR2* and *TLR4* THP-1 KD cells (Supplementary Figure 2*E*). While no changes in cell viability were observed (Supplementary Figure 2*F*), both KD cells produced significantly less IL-36γ (Figure 2*D*) upon infection.

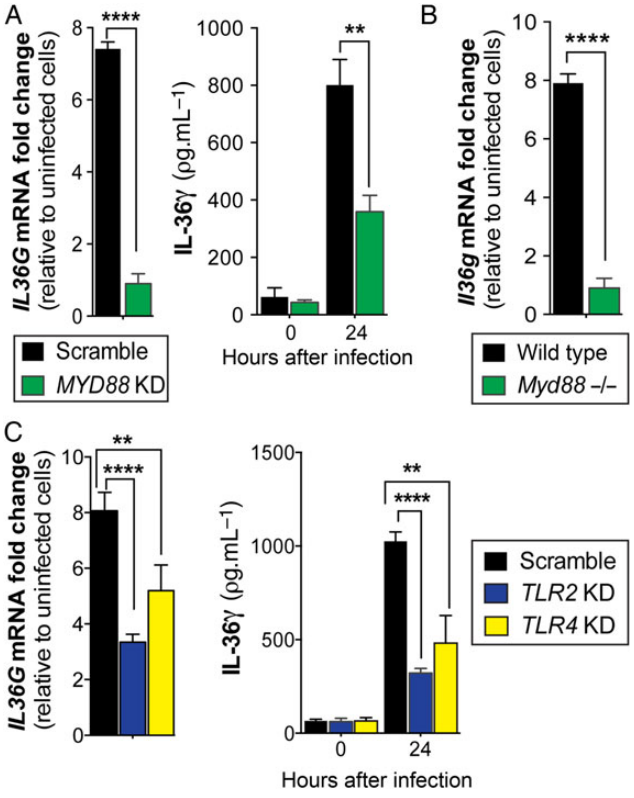

Figure 2. MyD88- and Toll-like receptor 2 (TLR2)/TLR4-dependent interleukin 36γ (IL-36γ) production upon *Mycobacterium tuberculosis* infection. *A*, IL-36γ messenger RNA (mRNA; 4 hours after infection) and protein (24 hours after infection) expression upon *M. tuberculosis* infection. *B*, IL-36γ mRNA (4 hours after infection) expression upon *M. tuberculosis* infection of bone marrow–derived macrophages (BMDMs) from wild-type or Myd88$^{-/-}$ knockout mice. *C*, Induction of IL-36γ mRNA (4 hours after infection) and protein (24 hours after infection) in *M. tuberculosis*–infected KD THP-1 macrophages. Data are representative of 4 independent experiments. $^{**}P \leq .01$, $^{***}P \leq .001$, and $^{****}P \leq .0001$.

M. tuberculosis Lipoproteins/Lipoglycans and Heat Shock Proteins (Hsps) Induce IL-36γ Upregulation via TLR2 and TLR4

We next identified PAMPs from *M. tuberculosis* involved in IL-36γ induction. To this end, we stimulated macrophages with different *M. tuberculosis*–derived fractions. Whole-cell lysate, γ-irradiated cell lysate, cell wall, and cell membrane fractions induced IL-36γ expression 4 hours (Figure 3*A*) and 24 hours (Supplementary Figure 3*A*) after stimulation. Using purified ligands, we found that lipoproteins, lipoarabinomannan, lipomannan, and Hsps, but not 6-kDa early secreted antigen target, mycolic acid-arabinogalactan-peptidoglycan (mAGP), and phosphatidylinositol mannoside, induced IL-36γ mRNA (Figure 3*B*). A possible influence of endotoxin contamination on IL-36γ induction via TLR4 was excluded, with only minor contamination detected in the Hsp65 fraction only (Supplementary Figure 3*B*). Heat inactivation of Hsp65, however, significantly reduced the induction of IL-36γ (Supplementary Figure 3*C*), verifying the stimulatory activity of this ligand.

To further analyze the TLR dependency of IL-36γ induction by *M. tuberculosis* ligands, we stimulated *TLR2* and *TLR4* KD macrophages. As additional controls for TLR2 or TLR4 stimulation, PAM3CSK4 and FSL1 or *E. coli* lipopolysaccharide, respectively, were used. *TLR2* KD abrogated IL-36γ stimulation by *M. tuberculosis*–derived lipoproteins and lipoglycans, whereas *TLR4* KD abolished the induction by several Hsps (Figure 3*C*).

Caspase-1 and Inflammasome Contribute to Amplification of IL-36γ Production

Caspase-1 and inflammasome play a central role in the regulation of IL-1β and IL-18 [10–12]. We interrogated whether this multiprotein complex also plays a role in regulating the expression of IL-36γ, another member of the IL-1 and IL-18 family. Macrophages preincubated with either pan-caspase inhibitor (Z-VAD-FMK) or caspase-1 inhibitor (Z-YVAD-FMK) released less IL-36γ 24 hours after infection with *M. tuberculosis* (Figure 4*A*). This was not due to differences in cell viability (Supplementary Figure 4*A*). Reduced IL-36γ protein expression was also observed in primary human MDMs (Supplementary Figure 4*B*). As a control, release of

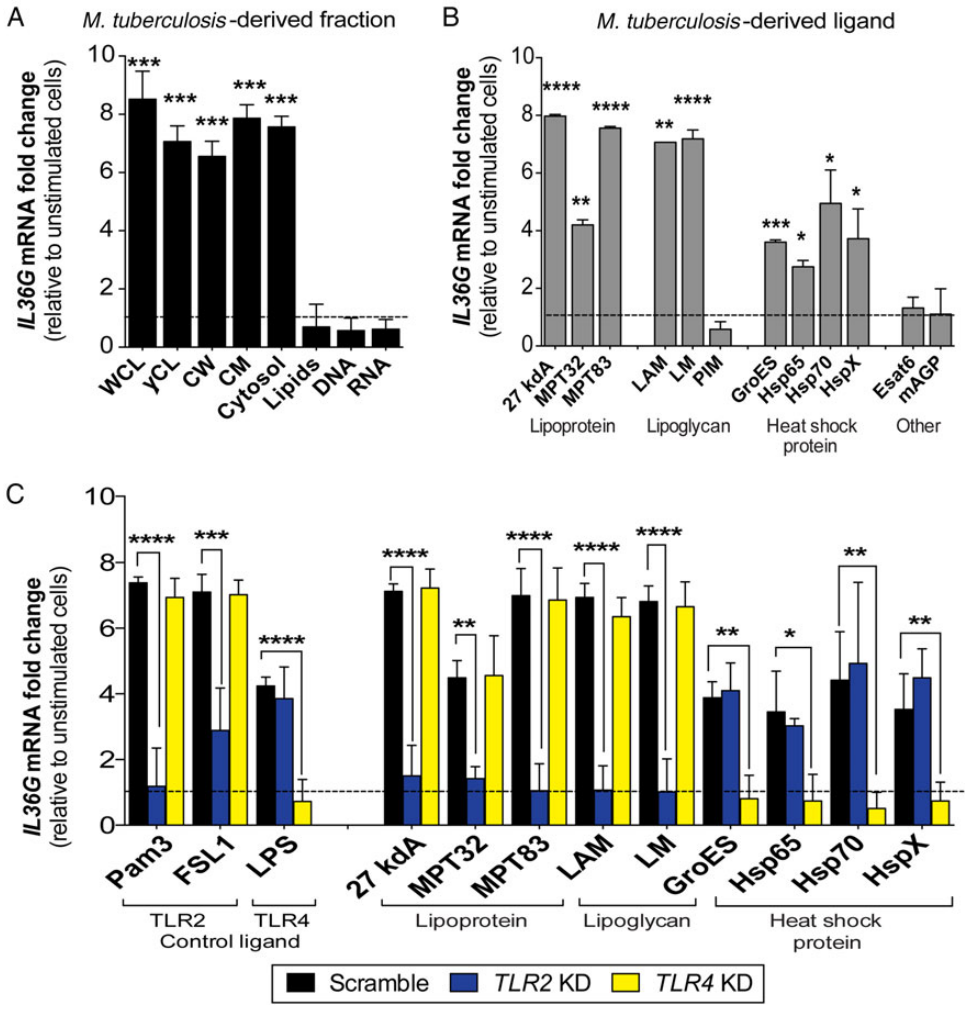

Figure 3. Induction of interleukin 36γ (IL-36γ) by *Mycobacterium tuberculosis*–derived fractions and ligands. Induction of IL-36γ messenger RNA (mRNA) expression (at 4 hours after infection) in THP-1 macrophages stimulated with *M. tuberculosis*–derived fractions (*A*), purified *M. tuberculosis*–derived ligands (*B*), and in *TLR2* and *TLR4* KD macrophages (*C*). Data are representative of 3 independent experiments (see also Supplementary Figure 3). $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, and $^{****}P \leq .0001$. Abbreviations: CM, cell membrane; CW, cell wall; Esat6, early secretory antigenic target 6; FSL1, fibroblast-stimulating synthetic triacylated lipoprotein; γCL, γ-irradiated cell lysate; Hsp, heat shock protein; LAM, lipoarabinomannan; LM, lipomannan; LPS, lipopolysaccharide; mAGP, mycolylarabinogalactan-peptidoglycan; Pam3, Pam3CSK4 synthetic diacylated lipoprotein; PIM, phosphatidylinositol mannoside; TLR, Toll-like receptor; WCL, whole-cell lysate.

IL-1β from prestimulated cells was impaired (Supplementary Figure 4*C*). Similarly, KD cells for caspase-1 (*CASP1*) or the inflammasome adaptor *ASC* produced less IL-36γ 24 hours after infection (Supplementary Figure 4*D* and Figure 4*B*). Consistent with previous reports [13, 16, 35, 36], we observed CASP1 and ASC dependency for IL-1β production but not for IL-6 production (Figure 4*B*). In line with these findings, the intracellular protein abundance of IL-36γ in *M. tuberculosis*–infected *CASP1* KD macrophages was profoundly reduced (Figure 4*C*).

Inflammasome receptors NLRP3 and AIM2 have been implicated in *M. tuberculosis*–derived inflammasome activation, leading to the secretion of other IL-1 family members, such as IL-1β [14–16]. Here, secreted levels of not only IL-1β protein but also of IL-36γ protein were reduced in *M. tuberculosis*–infected cells deficient of inflammasome receptors NLRP3 and AIM2 (Figure 4*D* and Supplementary Figure 4*E* and 4*F*). No differences in cell viability or IL-6 expression were observed (Supplementary Figure 4*G* and 4*H*).

IL-1β and IL-18 Are Sufficient for Caspase/Inflammasome-Dependent Induction of IL-36γ

Secretion of IL-1 family cognates is strongly regulated by a variety of proteolytic processing machineries, such as the caspase-inflammasome complex [11]. Since IL-36γ has been proposed to be cleaved in its N-terminal region [17], we analyzed predicted cleavage sites for CASP1 in the IL-36γ precursor. However, we did not identify any predicted CASP1 cleavage sites in the N-terminal part of the IL-36γ protein, using an Expasy tool (available at: http://web.expasy.org/peptide_cutter/). Therefore, we focused on IL-1β and IL-18 as possible alternative

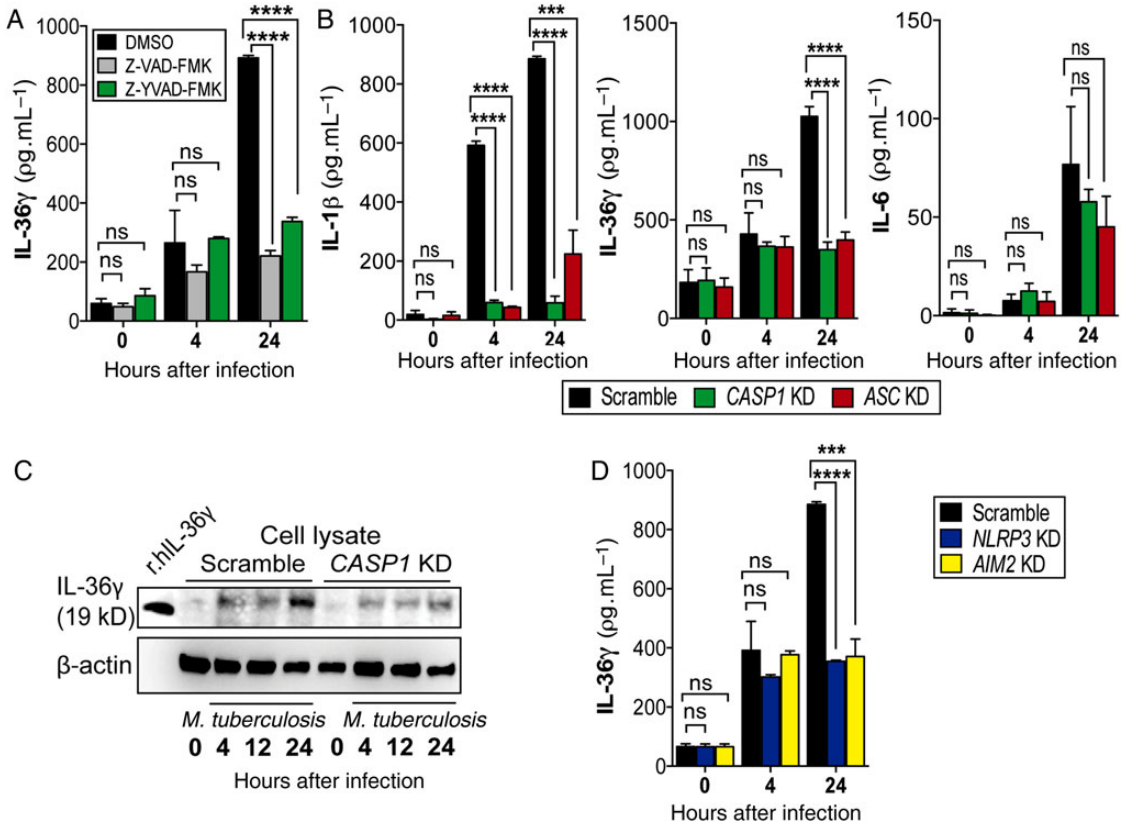

Figure 4. Caspase-1–inflammasome complex dependency for late-stage amplification of interleukin 36γ (IL-36γ) production. *A*, IL-36γ protein concentration in supernatants of infected THP-1 macrophages preincubated for 2 hours with Z-VAD-FMK/pan-caspase inhibitor or Z-YVAD-FMK/caspase-1 inhibitor. *B* and *C*, Concentration of interleukin 1β (IL-1β), IL-36γ, and interleukin 6 (IL-6) in supernatants (*B*) and intracellular IL-36γ protein from *Mycobacterium tuberculosis*–infected scramble and CASP1 KD cells (*C*). *D*, IL-36γ protein concentration in supernatants of *M. tuberculosis*–infected KD cells. Data are representative of at least 3 independent experiments. ***$P \leq .001$ and ****$P \leq .0001$. Abbreviations: DMSO, dimethyl sulfoxide; ns, not significant ($P > .05$).

inflammasome-dependent mediators regulating IL-36γ transcription. We analyzed IL-36γ mRNA expression in *CASP1* and *ASC* KD cells at different times after infection. Unexpectedly, we observed reduced IL-36γ mRNA levels at the later stage of *M. tuberculosis* infection (ie, ≥12 hours after infection) but not at earlier time points (Figure 5*A*). In contrast, IL-1β and IL-18 expression was already affected 4 hours after infection (Supplementary Figure 5*A* and 5*B*). Similar results were obtained with BMDMs from wild-type C57BL/6N mice and *Casp1*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice (Figure 5*A*).

IL-1β and IL-18 are potent inducers of IL-36γ [37]. Because secretion of both cytokines is inflammasome dependent [13, 16], we assessed whether these cytokines could account for the reduced IL-36γ expression as observed in CASP1-inflammasome–deficient cells. BMDMs from knockout mice deficient in *Il1b* or *Il18* were markedly impaired in IL-36γ induction upon *M. tuberculosis* infection (Figure 5*B*). We speculated that, in caspase-inflammasome–deficient cells, IL-36γ expression could be recovered by reconstitution with exogenous IL-1β or IL-18. Indeed, as shown in Figure 5*C* and 5*D* and Supplementary Figure 5*C*, both recombinant IL-1β and IL-18 reversed the phenotypes of *CASP1* and *ASC* KD cells and of *Casp1*$^{-/-}$ and *Casp1/11*$^{-/-}$ BMDMs, showing levels of IL-36γ expression similar to those for scrambled or wild-type controls.

IL-1R1 and IL-18R1 Are Essential for Late-Stage Amplification of IL-36γ Levels

To further validate the involvement of IL-1β and IL-18 in enhanced IL-36γ production, we targeted their respective receptors [11]. Blocking the IL-1 receptor with the antagonist anakinra strongly decreased production of IL-36γ at later time points (Figure 6*A* and 6*B* and Supplementary Figure 6*A*), whereas IL-6 levels were not affected (Supplementary Figure S6*B* and 6*C*). Use of KD cells for the IL-1β and IL-18 receptors (Supplementary Figure 6*D*) confirmed their involvement in IL-36γ production (Figure 6*C*). It is noteworthy that this effect could be restored by addition of alternate cytokines: recombinant IL-1β restored the effects of KD cells on *IL18R* signaling, and recombinant IL-18 restored the effects of KD cells on *IL1B* signaling (Figure 6*C*). Cell viability of KD cells was not affected (Supplementary Figure 6*E*). In sum, our findings suggest a feed-forward

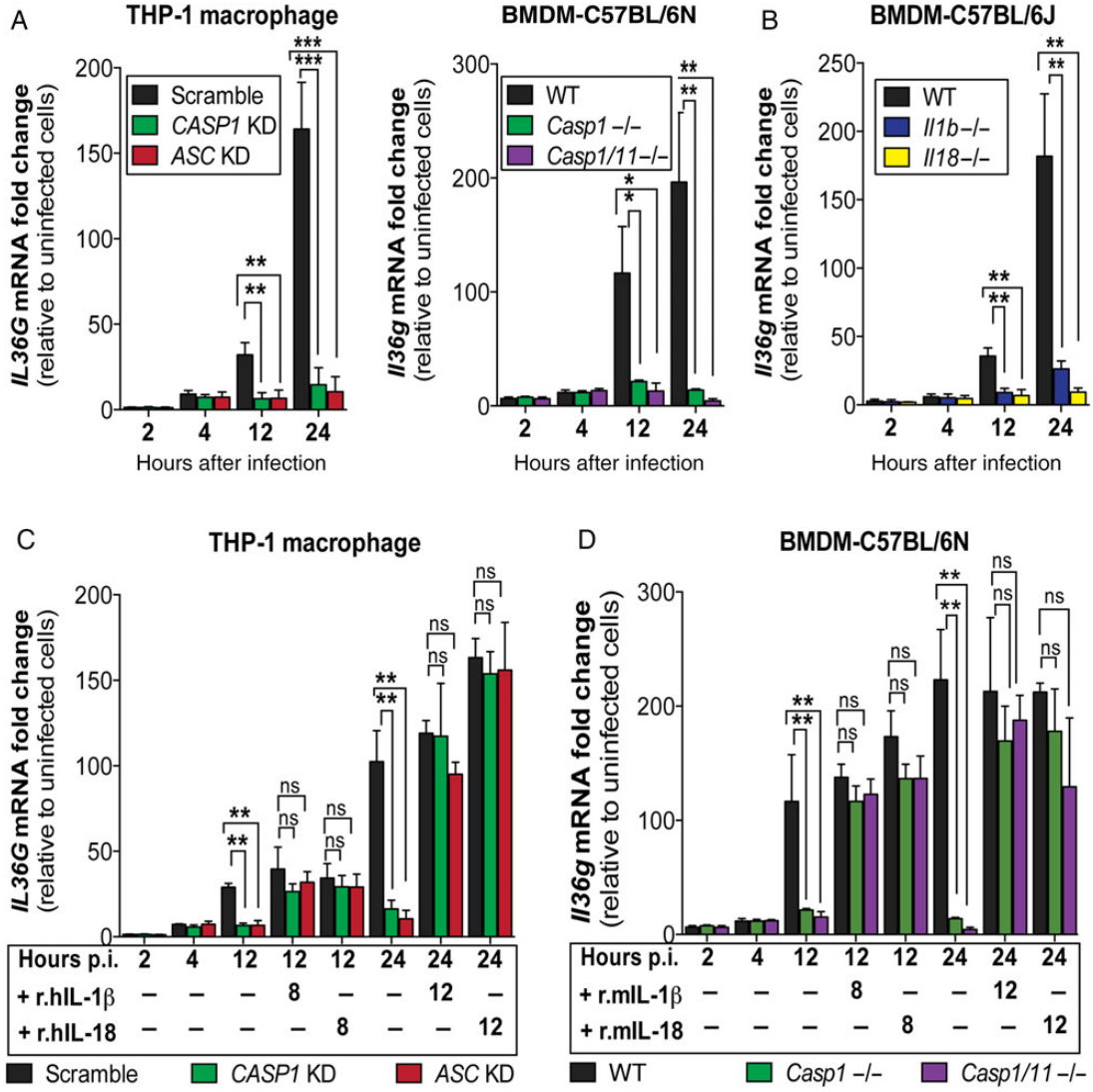

Figure 5. Amplification of interleukin 36γ (IL-36γ) upon *Mycobacterium tuberculosis* infection via interleukin 1β (IL-1β) and interleukin 18 (IL-18). *A* and *B*, Induction of IL-36γ expression in *M. tuberculosis*–infected THP-1 macrophages and bone marrow–derived macrophages (BMDMs) from *Casp1*$^{-/-}$ and *Casp1/11*$^{-/-}$ C57BL/6N mice (*A*) and *Il1b*$^{-/-}$ and *Il18*$^{-/-}$ C57BL/6J mice (*B*). *C* and *D*, Recombinant IL-1β and IL-18 reconstitution for 8 or 12 hours of infected (*C*) *CASP1* and *ASC* KD THP-1 macrophages and BMDMs from *Casp1*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice (*D*). All data are representative of 3 independent experiments. **P* ≤ .05, ***P* ≤ .01, ****P* ≤ .001, and *****P* ≤ .0001. Abbreviations: Hours p.i., hours post infection; mRNA, messenger RNA; ns, not significant (*P* > .05); r.hIL, recombinant human interleukin; r.mIL, recombinant murine interleukin; WT, wild type.

loop for IL-36γ amplification, mediated by IL-1β and IL-18 (Figure 6*D*).

IL-36 Mediates Antimicrobial Peptide Production and *M. tuberculosis* Growth Inhibition

Potent antimicrobial effects of IL-1β signaling through IL-1R1 have been described in tuberculosis [5, 8]. Hence, we interrogated whether the IL-36 pathway also confers antimicrobial effector mechanisms. We infected *IL36R* and *IL1R1* KD cells (Supplementary Figure 7*A*) and monitored cell viability and *M. tuberculosis* growth over time (Supplementary Figure 7*B*). *M. tuberculosis* load was significantly elevated 48 hours after infection and 5 days after infection in both *IL36R* and *IL1R1* KD macrophages, as determined by both CFU numbers (Figure 7*A* and Supplementary Figure 7*C*) and uracil uptake (Supplementary Figure 7*D*). Additionally, *M. tuberculosis* growth was elevated in *IL36R* KD cells preincubated with the IL-1R antagonist, reflecting a synergistic effect of IL-1β and IL-36 signaling in the control of *M. tuberculosis* growth (Figure 7*A* and Supplementary Figure 7*D*).

IL-36 cytokines induce APs such as beta defensins and cathelicidin in human keratinocytes [26, 27], and these APs have been shown to inhibit *M. tuberculosis* growth [28, 29, 38]. Stimulation of THP-1 macrophages with recombinant IL-36γ led to a dose-dependent upregulation of 15 selected human APs, including human cathelicidin (*CAMP*) and beta defensin 2

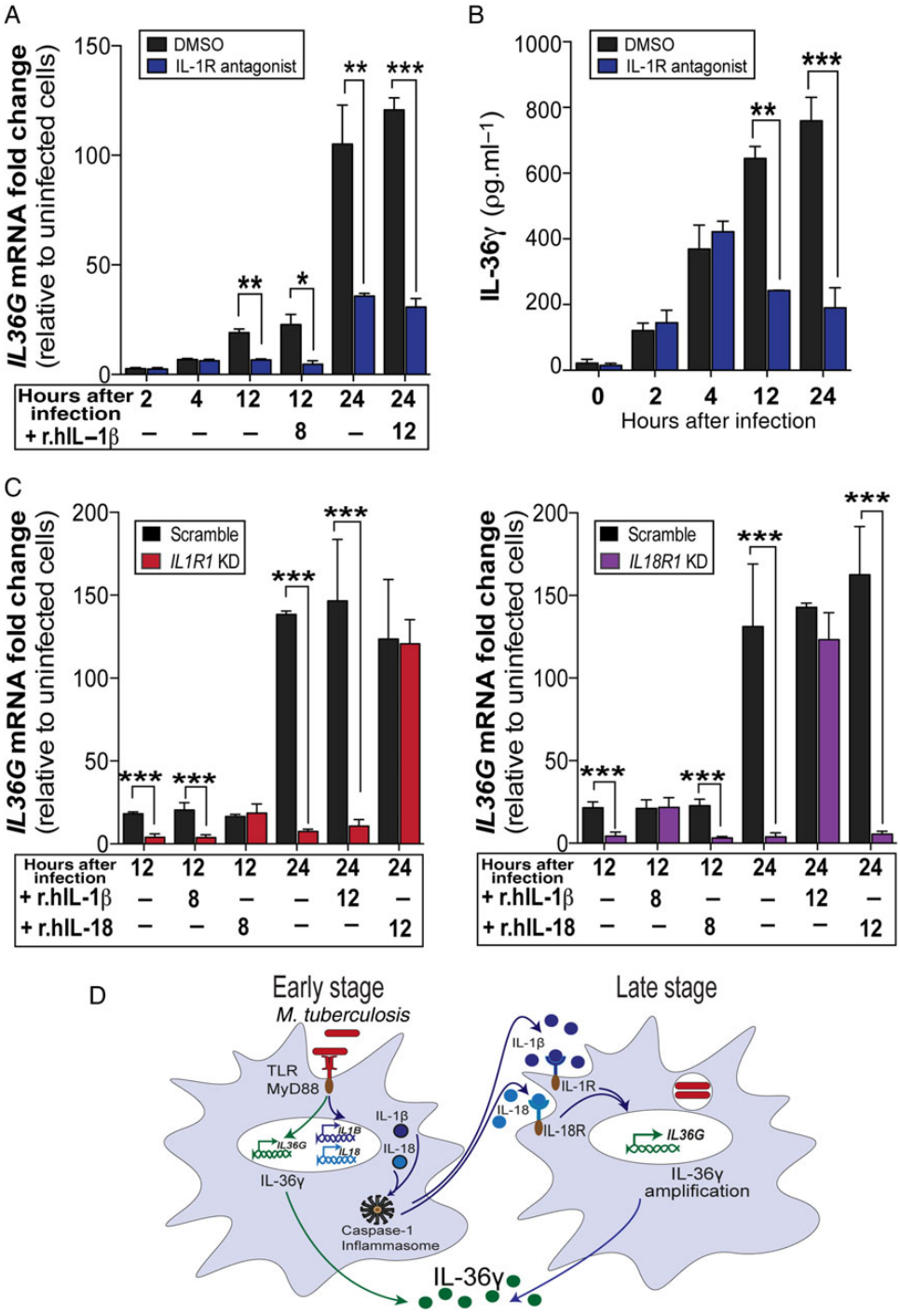

Figure 6. Interleukin 1R1 (IL-1R1) and interleukin 18R1 (IL-18R1) are essential for late-stage amplification of interleukin 36γ (IL-36γ) levels. *A* and *B*, Induction of IL-36γ messenger RNA (mRNA) (*A*) and protein secretion (*B*) from infected THP-1 macrophages after 3 hours of pretreatment with IL-1R antagonist (10 μM), with or without stimulation with recombinant human IL-1β (r.hIL-1β). *C*, IL-36γ induction in infected *IL1R1* KD and *IL18R1* KD cells stimulated with or without rhIL-1β or rhIL-18 for 8 or 12 hours. Data are representative of at least 2 independent experiments. *D*, Illustration of feed-forward model of IL-36γ induction in *Mycobacterium tuberculosis*–infected macrophage. $^{*}P \leq .05$, $^{**}P \leq .01$, $^{***}P \leq .001$, and $^{****}P \leq .0001$. Abbreviation: DMSO, dimethyl sulfoxide.

(*DEFB4*; Figure 7*B* and Supplementary Figure 7*D*). Protein expression of cathelicidin (hCAP18/LL37) and beta defensin 2 (hBD2) was also elevated by IL-36γ stimulation (Figure 7*C*). As controls, $1,25(OH)_2D3$ and IL-1β were used as inducers for cathelicidin (hCAP18) and beta defensin 2 (hBD2), respectively [28, 39]. Surprisingly, *IL36R* KD cells showed lower levels of both cathelicidin and beta defensin 2 upon *M. tuberculosis* infection, whereas only hBD2 expression was affected in

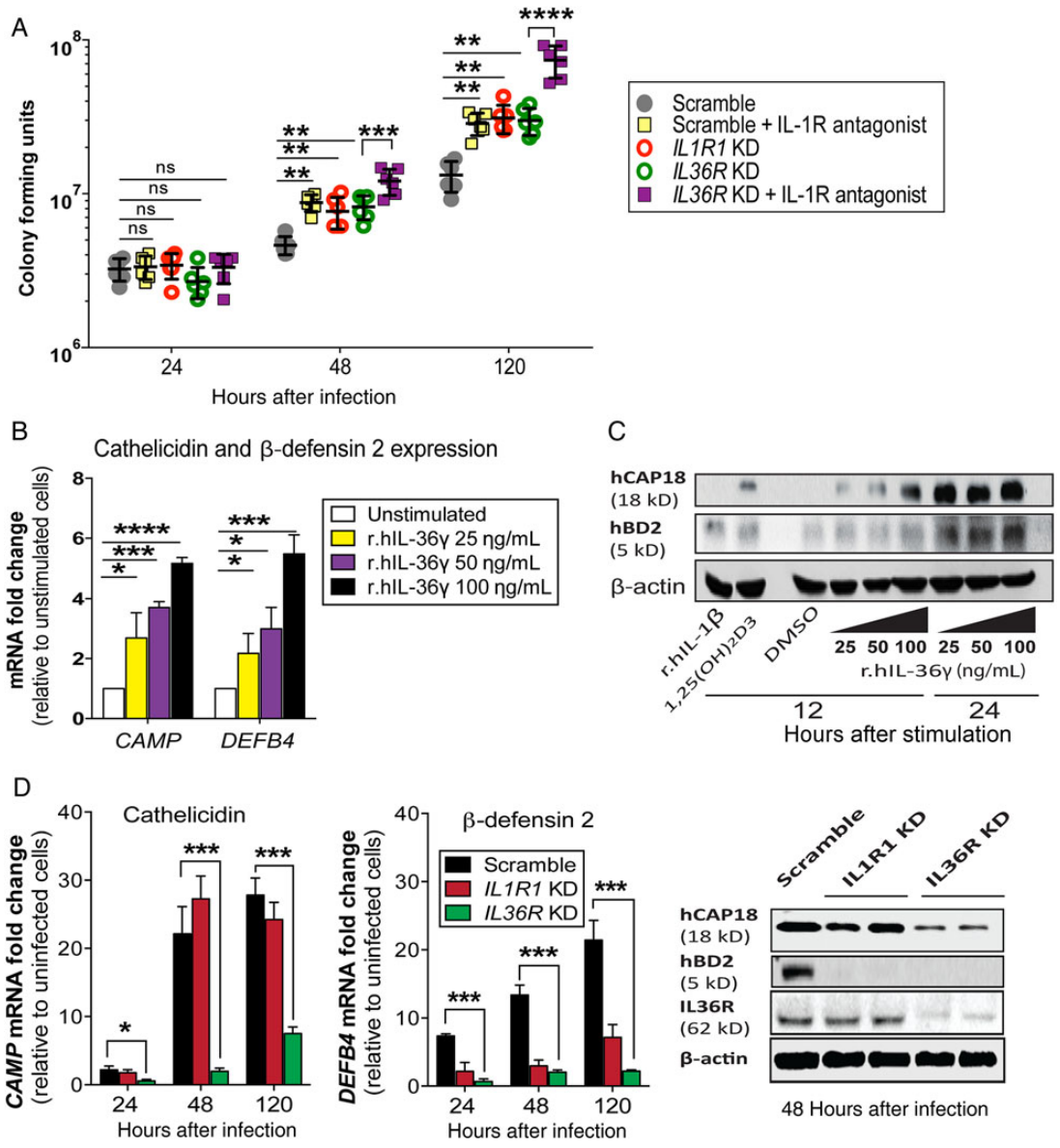

Figure 7. Interleukin 36 (IL-36) signaling induces antimicrobial peptides and restricts bacterial growth in *Mycobacterium tuberculosis*–infected macrophages. *A*, Quantification of viable bacteria from *M. tuberculosis*–infected KD cells, with or without pretreatment with the interleukin 1R (IL-1R) antagonist anakinra. Data are representative of 6 replicates from 4 independent experiments. *B*, Induction of *CAMP*/cathelicidin and *DEFB4*/beta defensin 2 messenger RNA (mRNA) expression from THP-1 macrophages stimulated for 4 hours with recombinant IL-36γ. *C*, Protein levels of hCAP18 (cathelicidin), hBD2 (beta defensin 2), and IL-36R in cell lysates from THP-1 macrophages stimulated with IL-36γ. Cells stimulated with recombinant human IL-β (r.hIL-1β; 50 ng/mL), 1,25(OH)$_2$D3 (50 nM), or dimethyl sulfoxide (DMSO) were included as controls. *D*, Induction of cathelicidin and beta defensin 2 in *M. tuberculosis*–infected KD THP-1 macrophages. Data are representative of 3 independent experiments. *$P \leq .05$, **$P \leq .01$, ***$P \leq .001$, and ****$P \leq .0001$. Abbreviations: ns, not significant; r.hIL, recombinant human interleukin.

IL1R1 KD cells (Figure 7*D* and Supplementary Figure 7*E*). Together, these results support a role of IL-36γ in the induction of APs that are capable of limiting *M. tuberculosis* growth.

DISCUSSION

Here we described a novel regulatory mechanism of IL-36γ induction relevant to the control of *M. tuberculosis*, involving initial PAMP-PRR activation in macrophages. We demonstrated that IL-36γ is induced by *M. tuberculosis* through TLR2 and TLR4 signaling, which is subsequently amplified by endogenous IL-1β and IL-18 in a caspase–inflammasome-dependent manner. We also provided evidence for an antimicrobial role of IL-36γ in host defense against *M. tuberculosis*, through regulation of AP expression and *M. tuberculosis* growth inhibition.

Although IL-36γ was discovered 15 years ago, its role and relevance in bacterial infections remains elusive [18, 40]. Previous studies have reported transcriptional upregulation of IL-36γ in human cells infected with rhinovirus and *Pseudomonas*

aeruginosa [41, 42]. Here we showed that *M. tuberculosis* infection of human cells induces IL-36γ, as well as other proinflammatory and antiinflammatory IL-36 cognates. We focused on the most strongly induced IL-36 family member, IL-36γ. Upon bacterial infection, various bacterial PAMPs are recognized by specific host PRRs, initiating the activation of different signaling pathways and culminating in the expression of immune genes, which determine the outcome of infection [5, 6]. We identified a variety of *M. tuberculosis* ligands that trigger IL-36γ production through TLR2 and TLR4 in a MyD88-dependent fashion. Our findings are in line with those of previous studies, showing that TLR2 senses lipoproteins, lipoarabinomannan, and lipomannan [43–46] and that TLR4 recognizes Hsps such as Hsp65 and Hsp70 [47]. We identified mycobacterial components, including secreted protein MPT32, which induced IL-36γ through TLR2, and GroES and HspX, which induced IL-36γ through TLR4. The involvement of TLR4 in IL-36γ induction is also in agreement with findings of a study showing induction of IL-36γ via TLR4 and dectin-1 signaling in human PBMCs infected with a fungal pathogen [19]. The recognition of *M. tuberculosis* by dectin-1 was first reported by Mahesh et al, revealing that virulent *M. tuberculosis* H37Rv induces the proinflammatory cytokines tumor necrosis factor α and IL-6 independent of dectin-1 [48]. To our knowledge, the exact nature of the dectin-1 ligand from *M. tuberculosis* has not yet been identified. A possible involvement of dectin-1 in IL-36γ induction by *M. tuberculosis*, however, was not addressed by this study. In addition, another important receptor for *M. tuberculosis* is NOD2. It has been reported that IL-36γ can be induced by the NOD2 agonist muramyl dipeptide in murine dendritic cells and that *M. tuberculosis*–derived mAGP can induce inflammatory cytokines partially dependent on NOD2 [49, 50]. In this study, we did not observe any induction of IL-36γ by *M. tuberculosis*–derived mAGP in human macrophages. A possible involvement of NOD2 in IL-36γ expression may differ between cell types and species. Although we do not exclude the involvement of other *M. tuberculosis* PAMPs and host PRRs, our findings demonstrate that TLR2, TLR4, and MyD88 are critically involved in *M. tuberculosis*–induced IL-36γ expression.

Here, we also identified a unique role of caspase-inflammasome complexes in the regulation of IL-36γ expression by demonstrating that inflammasome activation amplifies expression of IL-36γ via a feed-forward loop mediated by IL-1β and IL-18. Our model expands the functional relevance of caspase-inflammasome activation in cytokine regulation during microbial infection, involving crosstalk of IL-1 family members [18]. In addition, the IL-1β/IL-18 loop also substantiates the notion of alternative regulatory mechanisms by which the caspase-inflammasome complex can regulate the expression of cytokines that do not possess any apparent caspase-1 cleavage site.

So far, a protective role of IL-36γ against intracellular bacteria has been restricted to the induction of adaptive T-helper cell type 1 responses [20, 21]. Here, we add that IL-36γ also directly contributes to *M. tuberculosis* growth inhibition in human macrophages, likely by triggering AP production. In this study, we primarily focused our investigations on IL-36γ. Other experiments will need to be done to clarify whether other IL-36 isoforms have similar functions. Furthermore, murine *M. tuberculosis* infection experiments are being planned to determine the biological and antimicrobial effects of IL-36γ in an in vivo setting.

In summary, we have identified a unique host regulatory mechanism leading to IL-36γ production upon *M. tuberculosis* infection, which later induces generation of APs and restricts *M. tuberculosis* growth.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank Genentech and Anca Dorhoi, for kindly providing the *Casp1/11*$^{-/-}$ knockout mice; Erik Leif Sander and Charles Dinarello, for critical discussions; and Pau Morey, for providing the AP antibodies.

F. A. primarily performed the experiments with technical support of U. G.-B. and M. K.; F. A., J. M., and P. M.-A. designed the study, analyzed the data, and wrote the manuscript with major input from S. H. E. K.; P. M.-A., U. G.-B., and M. K. performed the KD cells generation; F. A. and P. M.-A. prepared the figures; and J. M. and S. H. E. K. conceived and supervised the project. F. A. and P. M.-A. equally contributed to the paper.

Financial support. This work was supported by the Max Planck Society.

Potential conflicts of interest. All authors: No potential conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. WHO. Global tuberculosis report. 20th ed. Geneva, Switzerland: World Health Organization, **2015**.
2. Dorhoi A, Kaufmann SH. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. Semin Immunopathol **2015**; 38:153-66.
3. Dorhoi A, Kaufmann SH. Versatile myeloid cell subsets contribute to tuberculosis-associated inflammation. Eur J Immunol **2015**; 45:2191–202.
4. Torrado E, Cooper AM. Cytokines in the balance of protection and pathology during mycobacterial infections. Adv Exp Med Biol **2013**; 783:121–40.
5. Fremond CM, Togbe D, Doz E, et al. IL-1 receptor-mediated signal is an essential component of MyD88-dependent innate response to *Mycobacterium tuberculosis* infection. J Immunol **2007**; 179:1178–89.
6. Fremond CM, Yeremeev V, Nicolle DM, Jacobs M, Quesniaux VF, Ryffel B. Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88. J Clin Invest **2004**; 114:1790–9.
7. Etna MP, Giacomini E, Severa M, Coccia EM. Pro- and anti-inflammatory cytokines in tuberculosis: a two-edged sword in TB pathogenesis. Semin Immunol **2014**; 26:543–51.
8. Mayer-Barber KD, Andrade BB, Oland SD, et al. Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature **2014**; 511:99–103.
9. Schneider BE, Korbel D, Hagens K, et al. A role for IL-18 in protective immunity against *Mycobacterium tuberculosis*. Eur J Immunol **2010**; 40:396–405.
10. Kurenuma T, Kawamura I, Hara H, et al. The RD1 locus in the *Mycobacterium tuberculosis* genome contributes to activation of caspase-1 via induction of potassium ion efflux in infected macrophages. Infect Immun **2009**; 77:3992–4001.

11. Afonina IS, Muller C, Martin SJ, Beyaert R. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. Immunity **2015**; 42: 991–1004.
12. van de Veerdonk FL, Netea MG, Dinarello CA, Joosten LA. Inflammasome activation and IL-1beta and IL-18 processing during infection. Trends Immunol **2011**; 32:110–6.
13. McElvania Tekippe E, Allen IC, Hulseberg PD, et al. Granuloma formation and host defense in chronic *Mycobacterium tuberculosis* infection requires PYCARD/ASC but not NLRP3 or caspase-1. PLoS One **2010**; 5:e12320.
14. Eklund D, Welin A, Andersson H, et al. Human gene variants linked to enhanced NLRP3 activity limit intramacrophage growth of *Mycobacterium tuberculosis*. J Infect Dis **2014**; 209:749–53.
15. Saiga H, Kitada S, Shimada Y, et al. Critical role of AIM2 in *Mycobacterium tuberculosis* infection. Int Immunol **2012**; 24:637–44.
16. Mishra BB, Moura-Alves P, Sonawane A, et al. *Mycobacterium tuberculosis* protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol **2010**; 12:1046–63.
17. Towne JE, Renshaw BR, Douangpanya J, et al. Interleukin-36 (IL-36) ligands require processing for full agonist (IL-36alpha, IL-36beta, and IL-36gamma) or antagonist (IL-36Ra) activity. J Biol Chem **2011**; 286:42594–602.
18. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity **2013**; 39:1003–18.
19. Gresnigt MS, Rosler B, Jacobs CW, et al. The IL-36 receptor pathway regulates Aspergillus fumigatus-induced Th1 and Th17 responses. Eur J Immunol **2013**; 43:416–26.
20. Vigne S, Palmer G, Martin P, et al. IL-36 signaling amplifies Th1 responses by enhancing proliferation and Th1 polarization of naive CD4+ T cells. Blood **2012**; 120:3478–87.
21. Foster AM, Baliwag J, Chen CS, et al. IL-36 promotes myeloid cell infiltration, activation, and inflammatory activity in skin. J Immunol **2014**; 192:6053–61.
22. Leemans JC, Thepen T, Weijer S, et al. Macrophages play a dual role during pulmonary tuberculosis in mice. J Infect Dis **2005**; 191:65–74.
23. Scordo JM, Knoell DL, Torrelles JB. Alveolar Epithelial Cells in *Mycobacterium tuberculosis* Infection: Active Players or Innocent Bystanders? J Innate Immun **2015**; 8:3–14.
24. Rivas-Santiago B, Hernandez-Pando R, Carranza C, et al. Expression of cathelicidin LL-37 during *Mycobacterium tuberculosis* infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. Infect Immun **2008**; 76:935–41.
25. Rivas-Santiago B, Schwander SK, Sarabia C, et al. Human {beta}-defensin 2 is expressed and associated with *Mycobacterium tuberculosis* during infection of human alveolar epithelial cells. Infect Immun **2005**; 73:4505–11.
26. Li N, Yamasaki K, Saito R, et al. Alarmin function of cathelicidin antimicrobial peptide LL37 through IL-36gamma induction in human epidermal keratinocytes. J Immunol **2014**; 193:5140–8.
27. Johnston A, Xing X, Guzman AM, et al. IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. J Immunol **2011**; 186:2613–22.
28. Liu PT, Schenk M, Walker VP, et al. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. PLoS One **2009**; 4: e5810.
29. Martineau AR, Wilkinson KA, Newton SM, et al. IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. J Immunol **2007**; 178:7190–8.
30. Raupach B, Peuschel SK, Monack DM, Zychlinsky A. Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against Salmonella enterica serovar Typhimurium infection. Infect Immun **2006**; 74:4922–6.
31. Shornick LP, De Togni P, Mariathasan S, et al. Mice deficient in IL-1beta manifest impaired contact hypersensitivity to trinitrochlorobenzone. J Exp Med **1996**; 183:1427–36.
32. Takeda K, Tsutsui H, Yoshimoto T, et al. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity **1998**; 8:383–90.
33. Kayagaki N, Warming S, Lamkanfi M, et al. Non-canonical inflammasome activation targets caspase-11. Nature **2011**; 479:117–21.
34. Akira S. Mammalian Toll-like receptors. Curr Opin Immunol **2003**; 15:5–11.
35. Mayer-Barber KD, Barber DL, Shenderov K, et al. Caspase-1 independent IL-1beta production is critical for host resistance to *mycobacterium tuberculosis* and does not require TLR signaling in vivo. J Immunol **2010**; 184:3326–30.
36. Kleinnijenhuis J, Joosten LA, van de Veerdonk FL, et al. Transcriptional and inflammasome-mediated pathways for the induction of IL-1beta production by *Mycobacterium tuberculosis*. Eur J Immunol **2009**; 39:1914–22.
37. Bachmann M, Scheiermann P, Hardle L, Pfeilschifter J, Muhl H. IL-36gamma/IL-1F9, an innate T-bet target in myeloid cells. J Biol Chem **2012**; 287:41684–96.
38. Klug-Micu GM, Stenger S, Sommer A, et al. CD40 ligand and interferon-gamma induce an antimicrobial response against *Mycobacterium tuberculosis* in human monocytes. Immunology **2013**; 139:121–8.
39. Verway M, Bouttier M, Wang TT, et al. Vitamin D induces interleukin-1beta expression: paracrine macrophage epithelial signaling controls *M. tuberculosis* infection. PLoS Pathog **2013**; 9:e1003407.
40. Dunn E, Sims JE, Nicklin MJ, O'Neill LA. Annotating genes with potential roles in the immune system: six new members of the IL-1 family. Trends Immunol **2001**; 22:533–6.
41. Chustz RT, Nagarkar DR, Poposki JA, et al. Regulation and function of the IL-1 family cytokine IL-1F9 in human bronchial epithelial cells. Am J Respir Cell Mol Biol **2011**; 45:145–53.
42. Vos JB, van Sterkenburg MA, Rabe KF, Schalkwijk J, Hiemstra PS, Datson NA. Transcriptional response of bronchial epithelial cells to Pseudomonas aeruginosa: identification of early mediators of host defense. Physiol Genomics **2005**; 21:324–36.
43. Doz E, Rose S, Nigou J, et al. Acylation determines the toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. J Biol Chem **2007**; 282:26014–25.
44. Gehring AJ, Dobos KM, Belisle JT, Harding CV, Boom WH. *Mycobacterium tuberculosis* LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. J Immunol **2004**; 173:2660–8.
45. Chen ST, Li JY, Zhang Y, Gao X, Cai H. Recombinant MPT83 derived from *Mycobacterium tuberculosis* induces cytokine production and upregulates the function of mouse macrophages through TLR2. J Immunol **2012**; 188:668–77.
46. Means TK, Lien E, Yoshimura A, Wang S, Golenbock DT, Fenton MJ. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. J Immunol **1999**; 163:6748–55.
47. Bulut Y, Michelsen KS, Hayrapetian L, et al. *Mycobacterium tuberculosis* heat shock proteins use diverse Toll-like receptor pathways to activate pro-inflammatory signals. J Biol Chem **2005**; 280:20961–7.
48. Yadav M, Schorey JS. The beta-glucan receptor dectin-1 functions together with TLR2 to mediate macrophage activation by mycobacteria. Blood **2006**; 108:3168–75.
49. Conforti-Andreoni C, Beretta O, Licandro G, et al. Synergism of NOD2 and NLRP3 activators promotes a unique transcriptional profile in murine dendritic cells. J Leukoc Biol **2010**; 88:1207–16.
50. Gandotra S, Jang S, Murray PJ, Salgame P, Ehrt S. Nucleotide-binding oligomerization domain protein 2-deficient mice control infection with *Mycobacterium tuberculosis*. Infect Immun **2007**; 75:5127–34.