

Role of Interleukin 36 γ 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 Maertzdorf¹

¹Department of Immunology, Max Planck Institute for Infection Biology, and ²ZIBI 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 36 γ (IL-36 γ), 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-36 γ 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-36 γ secretion. In the second stage, endogenous IL-1 β and interleukin 18 further amplify IL-36 γ synthesis. The relevance of this cytokine in the control of *M. tuberculosis* is demonstrated by IL-36 γ -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-36 γ during tuberculosis.

Keywords. IL-36 γ ; *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-1 β 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-1 β 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-36 α , IL-36 β , and IL-36 γ 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

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^aF. 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).

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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-36 γ in macrophages upon *M. tuberculosis* infection and its relevance in *M. tuberculosis* growth restriction. We uncover a dual-stage induction of IL-36 γ : an initial stage involving the recognition of *M. tuberculosis* PAMPs via TLRs and MyD88 pathways and a second regulatory stage in which endogenous IL-1 β and IL-18 further amplify the production of IL-36 γ . Furthermore, we have identified several APs that are induced in an IL-36 γ -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% CO₂. 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 Francisco, 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 OD₆₀₀ 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 × 10⁸ 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 Δ Ct 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-36 γ (Cusabo Biotech, Wuhan, China), IL-18 (MBL, Leuven, Belgium) and IL-1 β and IL-6 (both R&D Systems, 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 dH₂O 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-36 γ 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-36 α , IL-36 β , IL-36 γ , 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

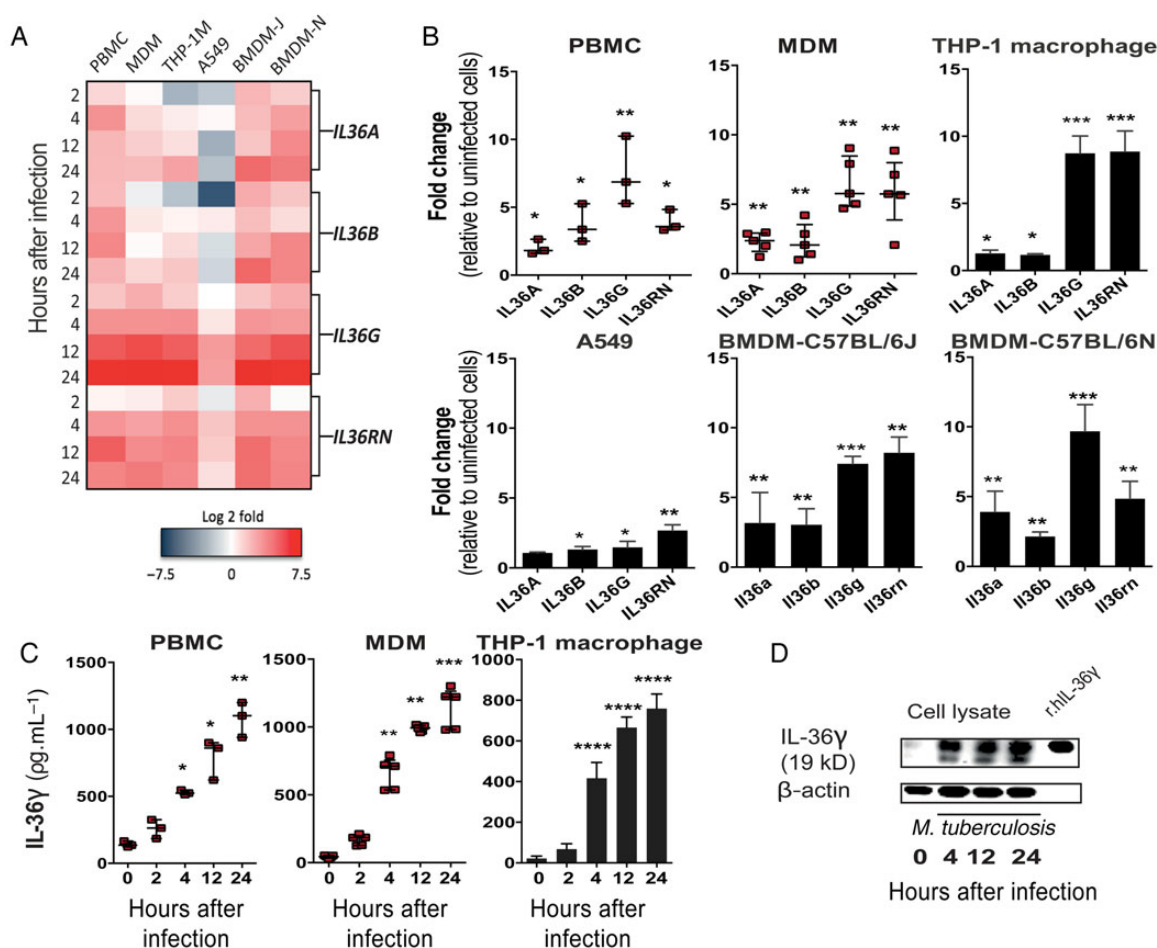


Figure 1. Interleukin 36 γ (IL-36 γ) induction upon *Mycobacterium tuberculosis* infection. *A*, Heat map of gene expression of IL-36 cognates (*IL36A*, *IL36B*, *IL36G*, and *IL36RN*) in different cells upon *M. tuberculosis* infection 2, 4, 12, and 24 hours after infection. Expression relative to uninfected 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-36 γ protein levels in supernatants of infected cells. *D*, Immunoblot of intracellular IL-36 γ from *M. tuberculosis*-infected THP-1 cell lysates. Recombinant human IL-36 γ (r.hIL-36 γ) 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* ≤ .05, ***P* ≤ .01, ****P* ≤ .001, and *****P* ≤ .0001. Abbreviation: THP-1 M, THP-1 macrophages.

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

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

Next we determined whether IL-36 γ production in response to *M. tuberculosis* infection is MyD88 dependent. To this end, we generated MYD88 KD macrophages, using small hairpin RNA (Supplementary Figure 2A). Upon *M. tuberculosis* infection, gene expression and secretion of IL-36 γ was significantly impaired in MYD88 KD THP-1 macrophages, as compared to scramble control cells (Figure 2A). Coherently, we found that BMDMs from *Myd88*^{-/-} mice failed to induce upregulation of IL-36 γ messenger RNA (mRNA; Figure 2B). No difference in cell viability was detected between KD and scramble control

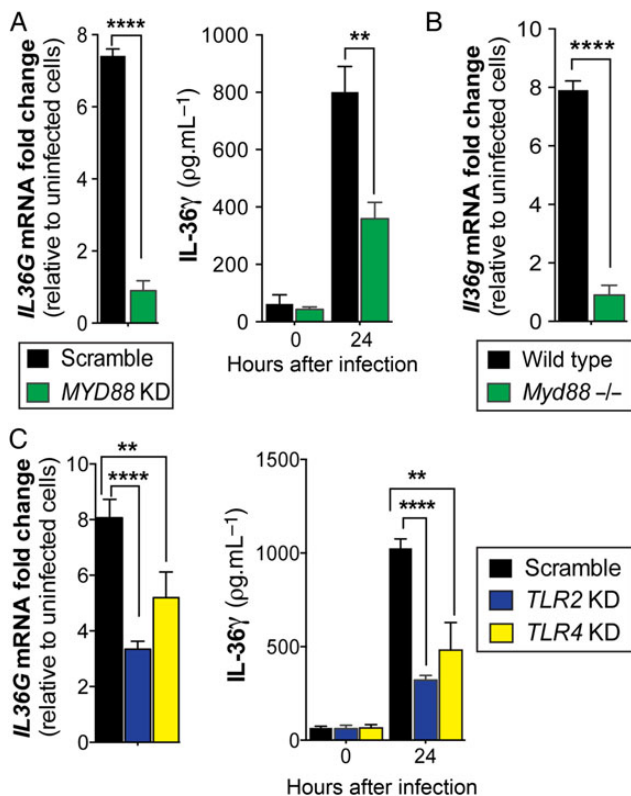


Figure 2. MyD88- and Toll-like receptor 2 (TLR2)/TLR4-dependent interleukin 36 γ (IL-36 γ) production upon *Mycobacterium tuberculosis* infection. A, IL-36 γ messenger RNA (mRNA; 4 hours after infection) and protein (24 hours after infection) expression upon *M. tuberculosis* infection. B, IL-36 γ mRNA (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-36 γ 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 **** $P \leq .0001$.

cells (Supplementary Figure 2B). These results suggest an involvement of MyD88-related TLRs in IL-36 γ production. To identify these, we analyzed several TLR agonists for their capacity to induce IL-36 γ expression [34]. TLR2 ligands, including Pam3CSK4, HKLM, and FSL1, as well as the TLR4 ligand lipopolysaccharide (derived from *Escherichia coli*), rapidly induced IL-36 γ mRNA (Supplementary Figure 2C). It is noteworthy that induction of IL-36 γ was independent from the presence of endotoxin in the ligands tested (Supplementary Figure 2D). To assess the TLR2 and TLR4 dependency of IL-36 γ induction by *M. tuberculosis*, we generated TLR2 and TLR4 THP-1 KD cells (Supplementary Figure 2E). While no changes in cell viability were observed (Supplementary Figure 2F), both KD cells produced significantly less IL-36 γ (Figure 2D) upon infection.

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

We next identified PAMPs from *M. tuberculosis* involved in IL-36 γ 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-36 γ expression 4 hours (Figure 3A) and 24 hours (Supplementary Figure 3A) 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-36 γ mRNA (Figure 3B). A possible influence of endotoxin contamination on IL-36 γ induction via TLR4 was excluded, with only minor contamination detected in the Hsp65 fraction only (Supplementary Figure 3B). Heat inactivation of Hsp65, however, significantly reduced the induction of IL-36 γ (Supplementary Figure 3C), verifying the stimulatory activity of this ligand.

To further analyze the TLR dependency of IL-36 γ 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-36 γ stimulation by *M. tuberculosis*–derived lipoproteins and lipoglycans, whereas TLR4 KD abolished the induction by several Hsps (Figure 3C).

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

Caspase-1 and inflammasome play a central role in the regulation of IL-1 β and IL-18 [10–12]. We interrogated whether this multi-protein complex also plays a role in regulating the expression of IL-36 γ , 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-36 γ 24 hours after infection with *M. tuberculosis* (Figure 4A). This was not due to differences in cell viability (Supplementary Figure 4A). Reduced IL-36 γ protein expression was also observed in primary human MDMs (Supplementary Figure 4B). As a control, release of

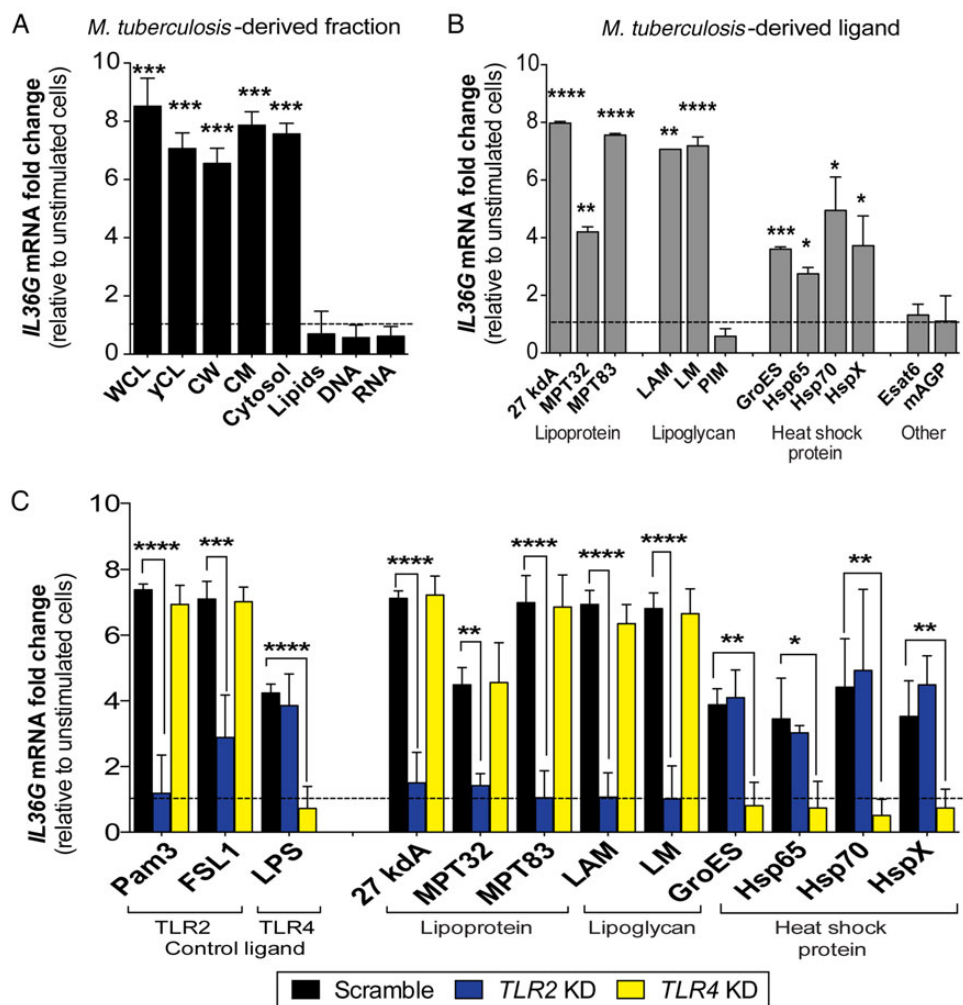


Figure 3. Induction of interleukin 36 γ (IL-36 γ) by *Mycobacterium tuberculosis*-derived fractions and ligands. Induction of IL-36 γ messenger RNA (mRNA) expression (at 4 hours after infection) in THP-1 macrophages stimulated with *M. tuberculosis*-derived fractions (A), purified *M. tuberculosis*-derived 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; γ CL, 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-1 β from prestimulated cells was impaired (Supplementary Figure 4C). Similarly, KD cells for caspase-1 (CASP1) or the inflammasome adaptor ASC produced less IL-36 γ 24 hours after infection (Supplementary Figure 4D and Figure 4B). Consistent with previous reports [13, 16, 35, 36], we observed CASP1 and ASC dependency for IL-1 β production but not for IL-6 production (Figure 4B). In line with these findings, the intracellular protein abundance of IL-36 γ in *M. tuberculosis*-infected CASP1 KD macrophages was profoundly reduced (Figure 4C).

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-1 β [14–16]. Here, secreted levels of not only IL-1 β protein but also of IL-36 γ protein were reduced in *M. tuberculosis*-infected cells deficient of inflammasome receptors NLRP3

and AIM2 (Figure 4D and Supplementary Figure 4E and 4F). No differences in cell viability or IL-6 expression were observed (Supplementary Figure 4G and 4H).

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

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-36 γ has been proposed to be cleaved in its N-terminal region [17], we analyzed predicted cleavage sites for CASP1 in the IL-36 γ precursor. However, we did not identify any predicted CASP1 cleavage sites in the N-terminal part of the IL-36 γ protein, using an ExPasy tool (available at: http://web.expasy.org/peptide_cutter/). Therefore, we focused on IL-1 β and IL-18 as possible alternative

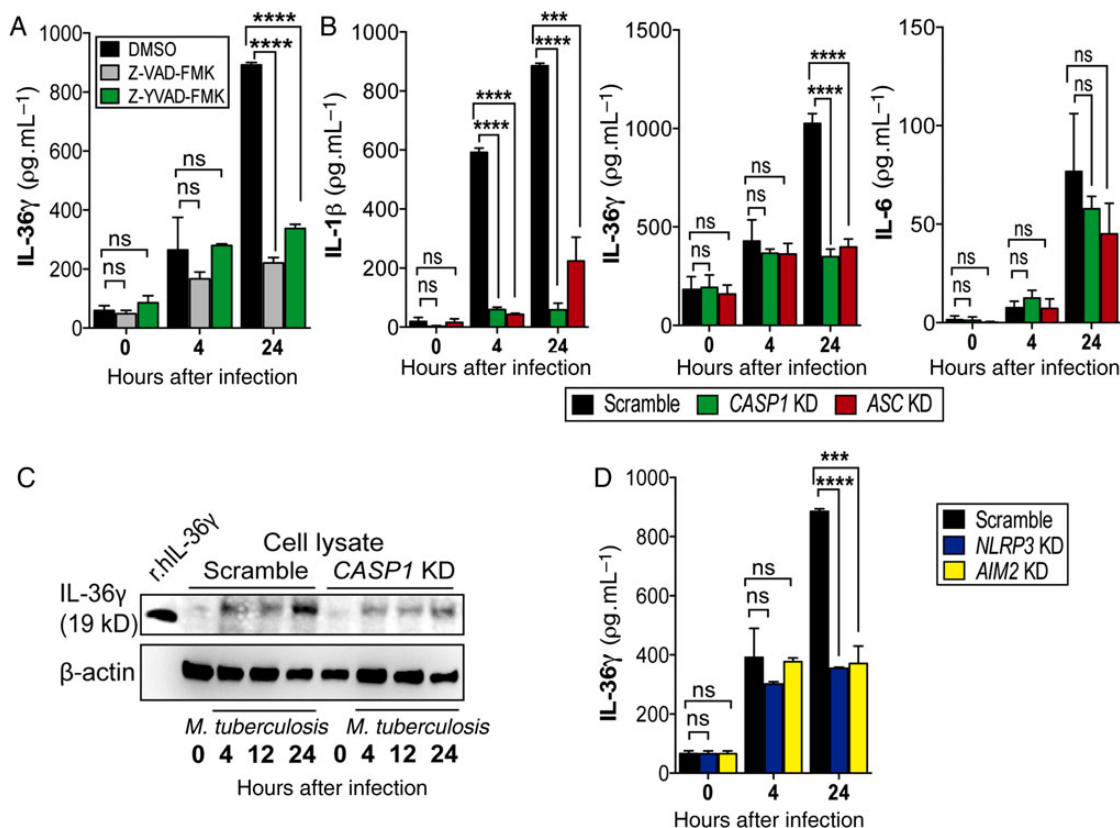


Figure 4. Caspase-1–inflammasome complex dependency for late-stage amplification of interleukin 36 γ (IL-36 γ) production. *A*, IL-36 γ protein concentration in supernatants of infected THP-1 macrophages preincubated for 2 hours with Z-VAD-FMK/pan-caspase inhibitor or Z-YVAD-FMK/caspase-1 inhibitor. *B* and *C*, Concentration of interleukin 1 β (IL-1 β), IL-36 γ , and interleukin 6 (IL-6) in supernatants (*B*) and intracellular IL-36 γ protein from *Mycobacterium tuberculosis*–infected scramble and CASP1 KD cells (*C*). *D*, IL-36 γ 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-36 γ transcription. We analyzed IL-36 γ mRNA expression in CASP1 and ASC KD cells at different times after infection. Unexpectedly, we observed reduced IL-36 γ mRNA levels at the later stage of *M. tuberculosis* infection (ie, ≥ 12 hours after infection) but not at earlier time points (Figure 5A). In contrast, IL-1 β and IL-18 expression was already affected 4 hours after infection (Supplementary Figure 5A and 5B). Similar results were obtained with BMDMs from wild-type C57BL/6N mice and *Casp1*^{-/-} and *Casp1/11*^{-/-} mice (Figure 5A).

IL-1 β and IL-18 are potent inducers of IL-36 γ [37]. Because secretion of both cytokines is inflammasome dependent [13, 16], we assessed whether these cytokines could account for the reduced IL-36 γ expression as observed in CASP1-inflammasome-deficient cells. BMDMs from knockout mice deficient in *Il1b* or *Il18* were markedly impaired in IL-36 γ induction upon *M. tuberculosis* infection (Figure 5B). We speculated that, in caspase-inflammasome-deficient cells, IL-36 γ expression could be recovered by reconstitution with exogenous IL-1 β or IL-18. Indeed, as shown in Figure 5C and 5D and Supplementary Figure 5C, both recombinant IL-1 β and

IL-18 reversed the phenotypes of CASP1 and ASC KD cells and of *Casp1*^{-/-} and *Casp1/11*^{-/-} BMDMs, showing levels of IL-36 γ expression similar to those for scrambled or wild-type controls.

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

To further validate the involvement of IL-1 β and IL-18 in enhanced IL-36 γ production, we targeted their respective receptors [11]. Blocking the IL-1 receptor with the antagonist anakinra strongly decreased production of IL-36 γ at later time points (Figure 6A and 6B and Supplementary Figure 6A), whereas IL-6 levels were not affected (Supplementary Figure S6B and 6C). Use of KD cells for the IL-1 β and IL-18 receptors (Supplementary Figure 6D) confirmed their involvement in IL-36 γ production (Figure 6C). It is noteworthy that this effect could be restored by addition of alternate cytokines: recombinant IL-1 β restored the effects of KD cells on *IL18R* signaling, and recombinant IL-18 restored the effects of KD cells on *IL1B* signaling (Figure 6C). Cell viability of KD cells was not affected (Supplementary Figure 6E). In sum, our findings suggest a feed-forward

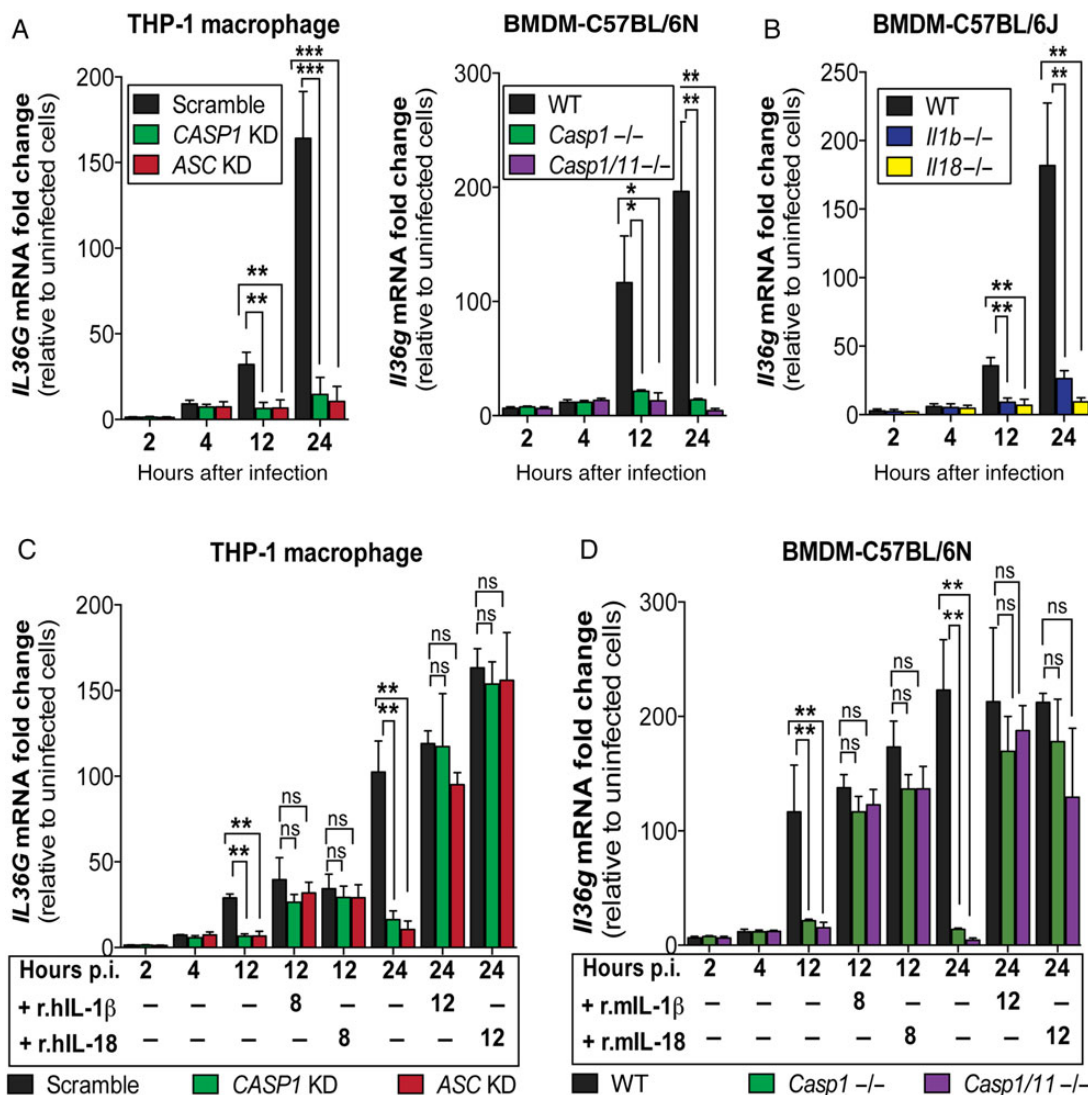


Figure 5. Amplification of interleukin 36 γ (IL-36 γ) upon *Mycobacterium tuberculosis* infection via interleukin 1 β (IL-1 β) and interleukin 18 (IL-18). *A* and *B*, Induction of IL-36 γ expression in *M. tuberculosis*-infected THP-1 macrophages and bone marrow-derived macrophages (BMDMs) from *Casp1*^{-/-} and *Casp1/11*^{-/-} C57BL/6N mice (*A*) and *Il1b*^{-/-} and *Il18*^{-/-} C57BL/6J mice (*B*). *C* and *D*, Recombinant IL-1 β 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-36 γ amplification, mediated by IL-1 β and IL-18 (Figure 6D).

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

Potent antimicrobial effects of IL-1 β 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 7A) and monitored cell viability and *M. tuberculosis* growth over time (Supplementary Figure 7B). *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 7A and Supplementary Figure 7C) and uracil uptake (Supplementary Figure 7D). Additionally, *M. tuberculosis* growth was elevated in *IL36R* KD cells preincubated with the IL-1R antagonist, reflecting a synergistic effect of IL-1 β and IL-36 signaling in the control of *M. tuberculosis* growth (Figure 7A and Supplementary Figure 7D).

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-36 γ led to a dose-dependent upregulation of 15 selected human APs, including human cathelicidin (*CAMP*) and beta defensin 2

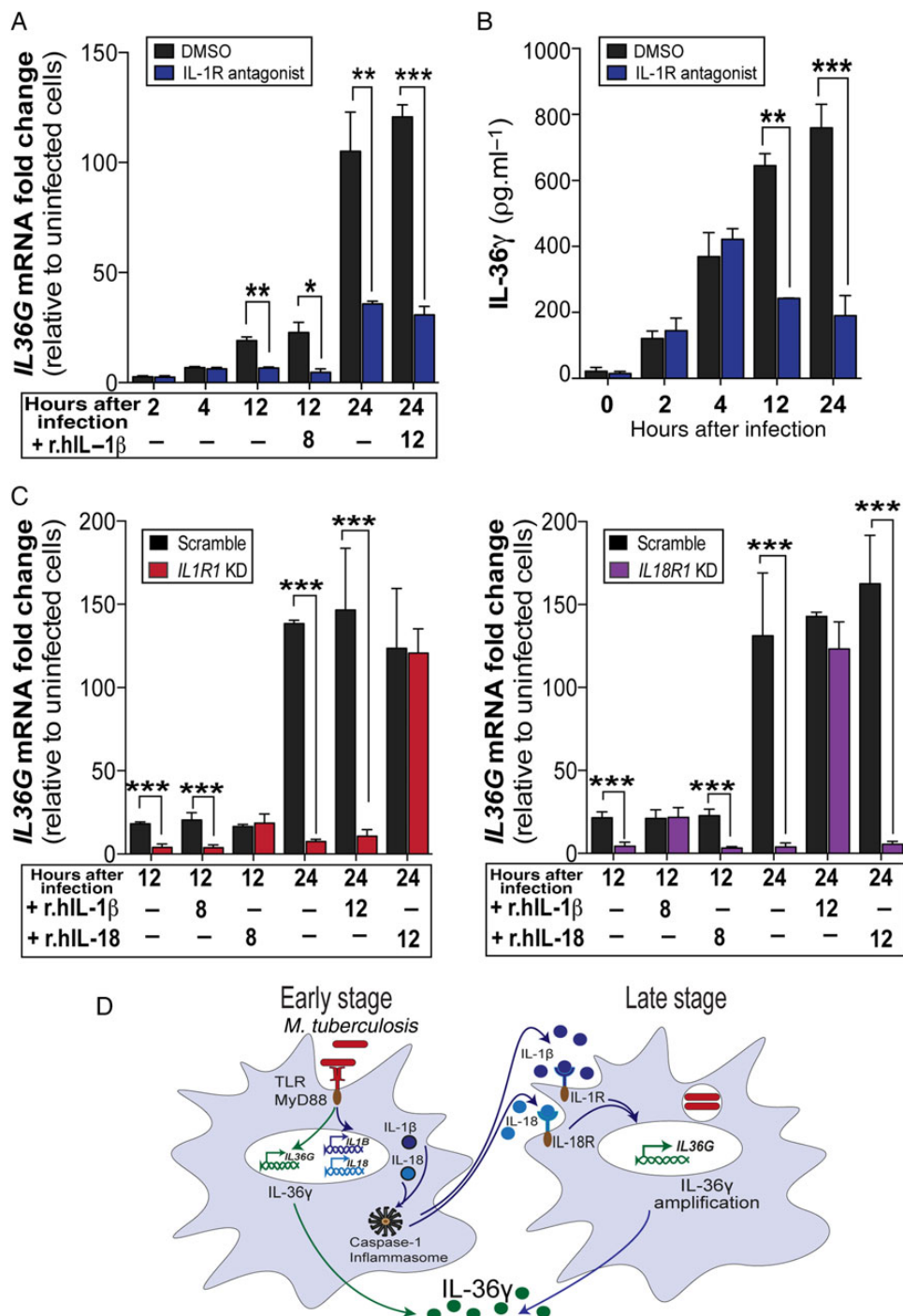


Figure 6. Interleukin 1R1 (IL-1R1) and interleukin 18R1 (IL-18R1) are essential for late-stage amplification of interleukin 36 γ (IL-36 γ) levels. *A* and *B*, Induction of IL-36 γ messenger RNA (mRNA) (*A*) and protein secretion (*B*) from infected THP-1 macrophages after 3 hours of pretreatment with IL-1R antagonist (10 μ M), with or without stimulation with recombinant human IL-1 β (r.hIL-1 β). *C*, IL-36 γ induction in infected *IL1R1* KD and *IL18R1* KD cells stimulated with or without rhIL-1 β 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-36 γ 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-36 γ stimulation (Figure 7*C*). As controls, 1,25(OH) $_2$ D3 and IL-1 β 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

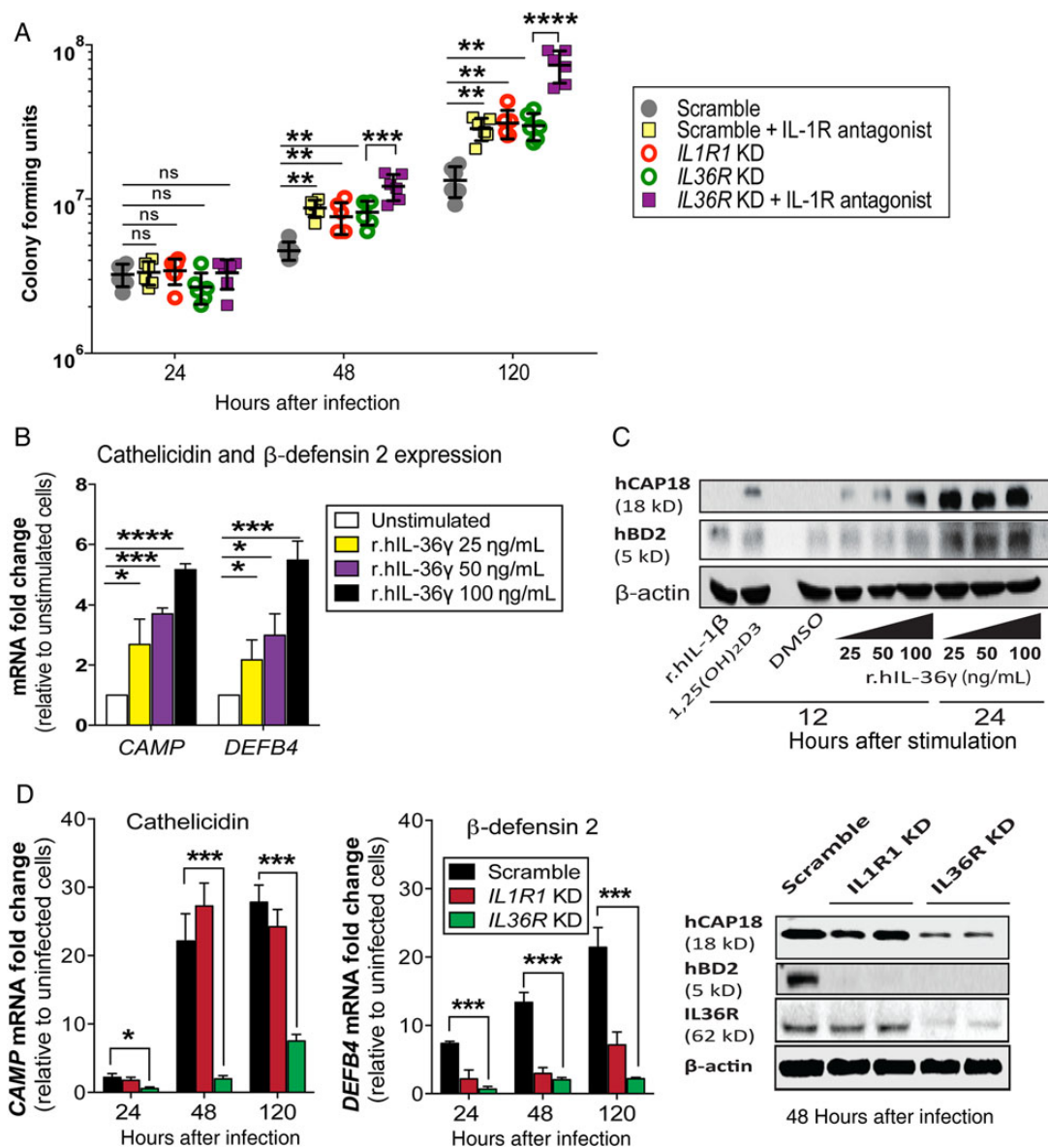


Figure 7. Interleukin 36 (IL-36) signaling induces antimicrobial peptides and restricts bacterial growth in *Mycobacterium tuberculosis*-infected macrophages. *A*, Quantification of viable bacteria from *M. tuberculosis*-infected KD cells, with or without pretreatment with the interleukin 1R (IL-1R) antagonist anakinra. Data are representative of 6 replicates from 4 independent experiments. *B*, Induction of *CAMP*/cathelicidin and *DEFBA4*/beta defensin 2 messenger RNA (mRNA) expression from THP-1 macrophages stimulated for 4 hours with recombinant IL-36 γ . *C*, Protein levels of hCAP18 (cathelicidin), hBD2 (beta defensin 2), and IL-36R in cell lysates from THP-1 macrophages stimulated with IL-36 γ . Cells stimulated with recombinant human IL-1 β (r.hIL-1 β ; 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 7D and Supplementary Figure 7E). Together, these results support a role of IL-36 γ in the induction of APs that are capable of limiting *M. tuberculosis* growth.

DISCUSSION

Here we described a novel regulatory mechanism of IL-36 γ induction relevant to the control of *M. tuberculosis*, involving initial PAMP-PRR activation in macrophages. We demonstrated that IL-36 γ is induced by *M. tuberculosis* through TLR2 and

TLR4 signaling, which is subsequently amplified by endogenous IL-1 β and IL-18 in a caspase-inflammasome-dependent manner. We also provided evidence for an antimicrobial role of IL-36 γ in host defense against *M. tuberculosis*, through regulation of AP expression and *M. tuberculosis* growth inhibition.

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

aeruginosa [41, 42]. Here we showed that *M. tuberculosis* infection of human cells induces IL-36 γ , as well as other proinflammatory and antiinflammatory IL-36 cognates. We focused on the most strongly induced IL-36 family member, IL-36 γ . 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-36 γ 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-36 γ through TLR2, and GroES and HspX, which induced IL-36 γ through TLR4. The involvement of TLR4 in IL-36 γ induction is also in agreement with findings of a study showing induction of IL-36 γ 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-36 γ 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-36 γ 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-36 γ by *M. tuberculosis*-derived mAGP in human macrophages. A possible involvement of NOD2 in IL-36 γ 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-36 γ expression.

Here, we also identified a unique role of caspase-inflammasome complexes in the regulation of IL-36 γ expression by demonstrating that inflammasome activation amplifies expression of IL-36 γ via a feed-forward loop mediated by IL-1 β 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-1 β /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-36 γ against intracellular bacteria has been restricted to the induction of adaptive T-helper cell

type 1 responses [20, 21]. Here, we add that IL-36 γ 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-36 γ . 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-36 γ in an in vivo setting.

In summary, we have identified a unique host regulatory mechanism leading to IL-36 γ 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

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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.

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