Title

IL-12+IL-18 Cosignaling in Human Macrophages and Lung Epithelial Cells Activates Cathelicidin and Autophagy, Inhibiting Intracellular Mycobacterial Growth

Journal

The Journal of Immunology, 200(7)

ISSN

0022-1767

Authors

Yang, Rui
Yang, Enzhuo
Shen, Ling
et al.

Publication Date

2018-04-01

DOI

10.4049/jimmunol.1701073

Peer reviewed

The Journal of Immunology

IL-12+IL-18 Cosignaling in Human Macrophages and Lung Epithelial Cells Activates Cathelicidin and Autophagy, Inhibiting Intracellular Mycobacterial Growth

Rui Yang, Enzhuo Yang, Ling Shen, Robert L. Modlin, Hongbo Shen and Zheng W. Chen

J Immunol 2018; 200:2405-2417; Prepublished online 16 February 2018;
doi: 10.4049/jimmunol.1701073
http://www.jimmunol.org/content/200/7/2405

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-12+IL-18 Cosignaling in Human Macrophages and Lung Epithelial Cells Activates Cathelicidin and Autophagy, Inhibiting Intracellular Mycobacterial Growth

Rui Yang,*,†,1 Enzhuo Yang,‡,1 Ling Shen,‡ Robert L. Modlin,§,¶ Hongbo Shen,* and Zheng W. Chen‡,‖

The ability of *Mycobacterium tuberculosis* to block host antimicrobial responses in infected cells provides a key mechanism for disease pathogenesis. The immune system has evolved to overcome this blockade to restrict the infection, but it is not clear whether two key innate cytokines (IL-12/IL-18) involved in host defense can enhance antimycobacterial mechanisms. In this study, we demonstrated that the combination of IL-12 and IL-18 triggered an antimicrobial response against mycobacteria in infected macrophages (THP-1 and human primary monocyte-derived macrophages) and pulmonary epithelial A549 cells. The inhibition of intracellular bacterial growth required p38–MAPK and STAT4 pathways, the vitamin D receptor, the vitamin D receptor–derived antimicrobial peptide cathelicidin, and autophagy, but not caspase-mediated apoptosis. Finally, the ability of IL-12+IL-18 to activate an innate antimicrobial response in human primary macrophages was dependent on the autonomous production of IFN-γ and the CAMP/autophagy pathway. Together, these data suggest that IL-12+IL-18 cosignaling can trigger the antimicrobial protein cathelicidin and autophagy, resulting in inhibition of intracellular mycobacteria in macrophages and lung epithelial cells. *The Journal of Immunology*, 2018, 200: 2405–2417.

As part of the first line of defense against *Mycobacterium tuberculosis*, the innate immune response involves macrophages and lung epithelial cells (1, 2). Within macrophages, *M. tuberculosis* can counteract the host defense via multiple strategies, resulting in its survival. In addition, *M. tuberculosis* infects pulmonary epithelial cells, resulting in the production of proinflammatory cytokines (e.g., TNF-α), chemokines (CXCL8, CXCL10, CCL5, and CCL2), NO, and antimicrobial peptides (β-defensin-2, cathelicidin), but the bacteria are not eliminated (3–5). Despite the development of an adaptive T cell response, the infection can progress. *M. tuberculosis* is estimated to have infected one third of the world's population and, as a result of its ability to evade the human immune response, kills >4000 people each day (6).

A better understanding of the host–*M. tuberculosis* interaction is required to develop improved intervention strategies to block transmission of *M. tuberculosis* and reactivation of disease in the already-infected population. A successful immune response against *M. tuberculosis* involves the distinct and overlapping contributions of the innate and adaptive immune systems, including the production of cytokines by the innate immune response, which instructs the adaptive T cell response (7, 8). For example, two key cytokines produced upon activation of the innate immune response by mycobacterial infection are IL-12 and IL-18 (9–16), which synergize to directly activate the adaptive T cell response toward Th1 cell differentiation (17). There is evidence that the combination of IL-12 and IL-18 can directly activate the innate immune system by inducing IFN-γ production in NK cells (18) and macrophages (19–21). Yet it is not known whether IL-12 plus IL-18 (IL-12+IL-18) directly triggers an antimicrobial response against *M. tuberculosis* in infected cells. To address this gap, we investigated whether IL-12 and IL-18 cooperate to directly activate a microbicidal pathway against intracellular mycobacteria in infected monocytes/macrophages, as well as pulmonary epithelial cells.

Our initial studies indicated that IL-12 or IL-18 alone had no effect on the viability of intracellular mycobacteria and that only cotreatment of infected macrophages with IL-12+IL-18 inhibited bacterial growth. Concomitantly, mRNAs encoding for the antimicrobial peptides CAMP and DEFB4A, as well as protein levels of CAMP, were induced by IL-12+IL-18 treatment. The inhibition of intracellular mycobacteria growth in A549 and THP-1 cells mediated by IL-12+IL-18 was blocked by lentiviral transduction vectors knocking down the expression of the vitamin D receptor (VDR). Moreover, inhibition of the p38–MAPK or STAT4 signaling pathway, as well as autophagy, in bacillus Calmette–Guérin (BCG)-infected IL-12+IL-18–treated cells abrogated the IL-12+IL-18 inhibition of intracellular mycobacteria growth. Furthermore,

*Unit of Anti-Tuberculosis Immunity, Key Laboratory of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200031, China; †University of Chinese Academy of Sciences, Beijing 100039, China; ‡Department of Microbiology and Immunology, Center for Primate Biomedical Research, University of Illinois College of Medicine, Chicago, IL 60612; §Department of Microbiology, Immunology and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095; ¶Division of Dermatology, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA 90095; and ‖Institut Pasteur of Shanghai, Shanghai 200031, China

[1]R.Y. and E.Y. contributed equally to this work and share first authorship.

ORCIDs: 0000-0001-7679-9646 (R.Y.); 0000-0001-9131-482X (E.Y.); 0000-0003-4720-031X (R.L.M.).

Received for publication July 26, 2017. Accepted for publication January 25, 2018.

This work was supported by the National Key Research and Development Program of China (Grant 2016YFA0502204), the National Institutes of Health (Grants R01 OD015092/RR13601, R01 HL64560, and R01 HL129887), and the National Program Project (Grant 2013ZX10003009-002) (all to Z.W.C.).

Address correspondence and reprint requests to Dr. Hongbo Shen, Institute Pasteur of Shanghai, Life Science Research Building, 320 Yueyang Road, Shanghai 200031, China. E-mail address: hbshen@ips.ac.cn

The online version of this article contains supplemental material.

Abbreviations used in this article: BCG, bacillus Calmette–Guérin; BSL3, biosafety level-3; hMDM, human monocyte-derived macrophage; MOI, multiplicity of infection; RT-qPCR, real-time quantitative PCR; shRNA, short hairpin RNA; TB, tuberculosis; VDR, vitamin D receptor.

IL-12+IL-8 treatment induces IFN-γ–dependent killing of intracellular mycobacteria in human primary macrophages. These data support the view that IL-12 and IL-18 cooperate to trigger an innate immune response that, via the autonomous production of IFN-γ, inhibits the growth of intracellular mycobacteria in human macrophages.

Materials and Methods

Ethics statement

The protocols for use of human blood samples for in vitro experimental procedures were evaluated and approved by the institutional review boards for human donors' research and institutional biosafety committees at Institut Pasteur of Shanghai and the University of Illinois–Chicago College of Medicine. All studies were consistent with guidelines of Office for Human Research Protections. All subjects were anonymized adults and provided written informed consent.

Cells and reagents

The human monocyte cell line, THP-1, and the human lung adenocarcinoma epithelial cell line, A549, were grown in RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), and 10% heat-inactivated FBS (all from Life Technologies). Prior to infection, THP-1 cells were treated with 50 ng/ml PMA (Sigma-Aldrich) for 48 h to differentiate into macrophages and then were washed three times with prewarmed PBS and maintained in antibiotic-free media at 37˚C for further use. 293T cells (purchased from Cell Bank, Shanghai, China), used to produce lentivirus particles, were maintained in medium consisting of DMEM supplemented with high glucose (4.5 g/l), L-glutamine (2 mM), sodium pyruvate (1 mM), and 10% heat-inactivated FBS (all from Life Technologies) All cell lines in use were routinely tested for *Mycoplasma* infection using a 16s-based PCR, and new cultures were established monthly from frozen stocks.

Human PBMCs were isolated by density gradient centrifugation, using Ficoll-Paque PLUS medium (GE), from buffy coats prepared from the peripheral blood of healthy uninfected donors (Blood Center of Shanghai, Shanghai Hospital). Adherent monocytes were enriched from PBMCs by adherence on plastic culture plates for 2 h. Nonadherent cells were removed via vigorous washing three times using prewarmed PBS. Human monocyte–derived macrophages (hMDMs) were differentiated from adherent monocytes in RPMI 1640 medium, supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), 10% heat-inactivated FBS, and 50 ng/ml human M-CSF (Novoprotein), for 7 d.

Human IL-12 was purchased from Miltenyi Biotec (order number 130-096-704), and human IL-18 was purchased from BioVision (catalog number 4179-25); both cytokines were used at 50 ng/ml for stimulation. Human TNF-α was purchased from Invitrogen (50 ng/ml; catalog number 10602HNAE25), and human IFN-β was purchased from PeproTech (100 U/ml; catalog number 300-02BC). Neutralizing anti-human IL-18 mAb (clone 125-2H) and its isotype control (clone 11711), anti-human IL-12 (catalog number AF-219-NA) and its isotype control (catalog number AB-108), anti-human TNF-α (clone 28401) and its isotype control (clone MAB002), and anti-human IFN-γ (clone 25718) and its isotype control (clone MAB003) were purchased from R&D Systems. MAPK inhibitors (p38, SB203580, MEK1/2, and U0126-EtOH), NF-κB inhibitor (PDTC), autophagy inhibitors (3-MA and wortmannin), and apoptosis inhibitor (zVAD.fmk) (all from Beyotime) were used at 10 μM.

Bacteria culture, infection of cells, and measurement of intracellular mycobacterial growth

Mycobacterium bovis BCG, *Mycobacterium smegmatis*, and *M. tuberculosis* H37Rv were grown in Difco Middlebrook 7H9 Broth with 10% oleic acid–albumin–dextrose–catalase enrichment (Becton Dickinson), 0.05% (v/v) Tween 80, and 0.2% (v/v) glycerol, at 37˚C, as reported (22). Cells were infected with BCG at a multiplicity of infection (MOI) of 10 bacilli to 1 cell overnight. A549 cells were infected with *M. smegmatis* at an MOI of 1 bacillus to 1 cell for 4 h. hMDMs were infected with H37Rv at an MOI of 4 for 4 h. Postinfection, extracellular noninternalized bacilli were removed by washing with prewarmed PBS four times. Then, 5×10^4 mycobacteria-infected THP-1 cells, A549 cells, or hMDMs were cultured in multiple wells with 50 ng/ml of each cytokine or combinations in the presence or absence of 5 μg/ml neutralization Abs and their isotype controls, or inhibitors (inhibitors were incubated with cells for 60 min and then cytokines were added) in 200 μl of media without antibiotics in 96-well plates for 3 d. Then, wells were aspirated, and the infected cells were lysed in 200 μl of sterile PBS with 0.067% SDS. A 10-fold serial dilution was performed for quantitative culturing. Aliquots (100 μl) were plated in triplicate on Middlebrook 7H10 agar plates supplemented with 10% oleic acid–albumin–dextrose–catalase for 2–3 wk until colonies were large enough to be counted. Mycobacteria viability was quantified by counting CFU. The percentage survival index, represented as intracellular bacteria survival, was calculated as follows: survival index = 100 × CFU of treatment/CFU of media.

Quantification of gene expression

Total RNA was isolated from cells using a Direct-zol RNA Miniprep kit (Zymo) based on the spin columns method. Contaminating genomic DNA was removed using DNase I in columns, according to the manufacturer's protocols (Zymo). Then, RNA was reverse transcribed into cDNA using mixed oligod-dT18 and random hexamers primers with HiScript II Q RT SuperMix for qPCR (Vazyme). Real-time quantitative PCR (RT-qPCR) reactions were performed in triplicate in 384-well plates on an Applied Biosystems PRISM 7900HT Fast Real-Time PCR System using THUNDERBIRD qPCR Mix (Toyobo). Expression values were normalized to those of the housekeeping gene *EF1A*. Oligonucleotides used for amplification are listed in Table I and were synthesized by Sangon Biotech.

Generation of lentiviral–short hairpin RNA and pCDH-GFP-LC3 constructs, lentivirus preparation, and infection

The short hairpin RNA (shRNA) lentiviral construct (synthesized by Sangon Biotech) targeting VDR (5′-CTCCTGCCTACTCACGATAAA-3′), STAT4 (5′-GCGAGACTACAAAGTTATTAT-3′), CAMP (5′-GCTTCGTGCTATAGATGGCAT-3′), or control was cloned into pLKO.1 lentiviral vectors using restriction enzymes AgeI and EcoRI (NEB). GFP-LC3 was cloned from pEGFP-LC3 (human, plasmid number 24920; Addgene) and then introduced into pCDH-Puro backbone to create the pCDH–GFP–LC3 construct. 293T cells were transfected with the lentivirus constructs, pMD2G and pxPAS2 vectors, using HG-Trans293 transfection reagent (Yeasen), according to the manufacturer's protocol. After 24 h, media were discarded, and fresh media were added. At days 4 and 5, the cell suspension was collected, pooled, and filtered through a 0.45-μm filtration unit (Merck/Millipore). Then, the virus particles were concentrated using a GML-PC lentivirus concentration kit (Genomeditech), according to the manufacturer's protocol. A549 cells were treated with filtered viral supernatant and 8 μg/ml Polybrene (Yeasen). Then, virus was aspirated, and fresh media were added. THP-1 cells were infected by a spin-transduction through 800 × *g* for 2 h at 32˚C. Selection with puromycin (Yeasen) was started at 48 h after viral transduction, and the selection concentrations for A549 and THP-1 cells were 2 and 0.5 μg/ml, respectively.

Autophagy analysis

PMA-differentiated GFP-LC3–THP-1 cells were infected with BCG at an MOI of 10:1 overnight. Cells were then treated with media or IL-12+IL-18 for 12 h. For the quantitation of autophagy, the percentages of GFP-LC3$^+$ autophagic vacuoles in GFP-LC3–THP-1 cells were evaluated using fluorescence microscopy, as described (23).

Western blotting

Cells were lysed by incubation in RIPA lysis buffer, supplemented with 1 mM PMSF (Yeasen), on ice for 5 min. Then, cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck/Millipore). After blocking with 5% BSA in TBST for 2 h, the membrane was incubated with Ab against STAT4 (BBIAB; Sangon Biotech), VDR (Abcam), LC3 (Abcam), or GAPDH (BBIAB; Sangon Biotech) overnight at 4˚C, followed by incubation with the respective secondary Abs for 1 h. Protein bands were checked using ECL detection solution (Yeasen). In independent Western blot assays, cells for evaluating each of the targeted proteins were treated in duplicate or triplicate wells in a six-well plate in each of the experiments. Samples harvested from each well were run on separated SDS-PAGE gels, followed by transblotting and specific immune detection.

ELISA

The amounts of IFN-γ, TNF-α, and IL-32 in cell supernatants were detected using human cytokine ELISA kits (C608 for human IFN-γ; C609 for human TNF-α; C687 for human IL-32α, purchased from Beijing GenStar), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0. All data are expressed as mean ± SEM. Differences between groups were first assessed

by a nonparametric *t* test or ANOVA; the latter was followed by the Dunnett test or the Tukey multiple-comparison test, as indicated in the figure legends. The *p* values are adjusted for multiple comparisons (family-wise significance [confidence level of 0.05 and 95% confidence interval]).

Results

IL-12+IL-18 cotreatment of Mycobacterium*-infected cells from monocytic and lung epithelial lineages led to inhibition of intracellular mycobacterial growth*

To address whether IL-12 and/or IL-18 directly affected intracellular mycobacterial infection in monocytes/macrophages and lung epithelial cells, we used an in vitro human infection model (24, 25). Experiments were initiated in a biosafety level-2 laboratory using less virulent mycobacteria and then were performed using virulent *M. tuberculosis* H37Rv strain for confirmation in a biosafety level-3 (BSL3) laboratory. Mycobacteria-infected cells were treated for 72 h with media, recombinant human IL-12 alone, recombinant human IL-18 alone, or IL-12+IL-18 and then cells were lysed to release viable intracellular bacteria for measuring CFU counts on Middlebrook 7H10 agar plates. Thus, A549 cells, which usually serve as a model for studying the host response of human type II alveolar epithelial cells to intracellular pathogens (26), were infected with *M. smegmatis* and *M. bovis* BCG. To select the optimal combination of cytokines to limit intracellular bacteria, dose-response experiments were performed; a combination of 50 ng/ml each cytokine was shown a reduced BCG and *M. tuberculosis* burden (Supplemental Fig. 1A, 1B). Although IL-18RAP mRNA and protein were expressed in infected and uninfected A549 cells (Supplemental Fig. 2), cotreatment with IL-12 +IL-18, but not IL-12 or IL-18 alone, significantly reduced the growth of *M. smegmatis* in A549 cells (Fig. 1A). Approximately 40% inhibition of intracellular *M. smegmatis* viability was observed compared with medium control ($p < 0.0001$, Fig. 1A). IL-12+IL-18 also inhibited intracellular BCG growth in BCG-infected A549 cells (Fig. 1B).

We then tested whether cotreatment with IL-12+IL-18 inhibited the growth of mycobacteria in pulmonary epithelial cells, as well as in human macrophages. We first used PMA-matured THP-1 cells, which are often used as a model to study the immune response of human macrophages (27). Cotreatment of BCG-infected THP-1 macrophage cells with IL-12+IL-18 significantly decreased the growth of intracellular mycobacteria, with ∼50% inhibition compared with controls (Fig. 1C). We next examined whether IL-12+IL-18-triggered growth inhibition could also be detected in hMDMs. Similarly, IL-12+IL-18 cotreatment significantly reduced the growth of intracellular BCG in hMDMs, with a mean inhibition ∼ 40% compared with control treatment (Fig. 1D).

To validate the specificity of IL-12+IL-18 growth inhibition of intracellular mycobacteria, we performed blocking experiments using neutralizing Abs. In the presence of IL-12+IL-18, blockade of IL-12 or IL-18 signaling by neutralizing anti–IL-12 or anti–IL-18 Ab significantly reduced the intracellular growth of *M. smegmatis* and BCG in hMDMs compared with isotype controls (Fig. 1E). This was consistent with the finding that IL-12+IL-18 treatment upregulated *IL-12A*, *IL-12B*, and *IL-18* in BCG-infected hMDMs (Supplemental Fig. 1C). However, in the absence of exogenous IL-12+IL-18, neutralization of endogenous IL-12 and IL-18 in BCG-infected hMDM cultures did not affect intracellular mycobacteria growth (Supplemental Fig. 1D). Although anti–IL-12– or anti–IL-18–neutralizing Ab was unable to efficiently penetrate the cell membrane to block endogenous IL-12/IL-18, we cannot exclude the possibility that concentrations of endogenous IL-12 and IL-18 induced by mycobacterial infection were not high enough to inhibit extremely high levels of mycobacterial infection in cultures. Similar blocking effects by neutralizing anti–IL-12 or anti–IL-18 Ab were observed in the other three systems involving IL-12+IL-18 cotreatment of *M. smegmatis* or BCG in A549 epithelial cells and THP-1 monocytic cells (Supplemental Fig. 1E–G).

We also sought to examine whether the IL-12+IL-18–mediated intracellular inhibition of mycobacteria could also be detected at day 6 after the coculture. As seen in Fig. 1F, IL-12+IL-18 cotreatment significantly restricted intracellular BCG growth in A549 cells at day 3, as well as at day 6. Taken together, these data suggest that the simultaneous activation of IL-12 and IL-18 signaling pathways results in potent growth inhibition of intracellular mycobacteria in human monocytic and lung epithelial lineages.

Cotreatment of hMDMs with IL-12+IL-18 consistently inhibited intracellular growth of virulent M. tuberculosis *H37Rv*

We then sought to determine whether cotreatment of monocytes/macrophages with IL-12+IL-18 could also inhibit the intracellular growth of virulent *M. tuberculosis* bacilli as we demonstrated in the setting of *M. smegmatis* and BCG infection. To address this, we performed growth-inhibition experiments using the virulent *M. tuberculosis* H37Rv strain for infection of hMDMs under BSL3 conditions. Similarly, *M. tuberculosis* H37Rv–infected hMDMs were cultured with media or IL-12+IL-18 for 3 d, and CFU were counted on 7H11 agar plates. Consistent with the findings for *M. smegmatis* and BCG, treatment of *M. tuberculosis*–infected hMDMs with IL-12+IL-18 also led to significant and reproducible inhibition of intracellular H37Rv bacilli growth at day 3 (Fig. 2A, inhibition rate 39%, $p = 0.0296$). Moreover, IL-12+IL-18 treatment still showed a suppressive effect at day 6 (Fig. 2B). These results provided further support that IL-12+IL-18 could restrict the intracellular growth of mycobacteria, including virulent *M. tuberculosis*.

VDR activation of antimicrobial peptides appeared to be required for the IL-12+IL-18 cosignaling–mediated growth inhibition of intracellular mycobacteria

We then conducted studies to explore the mechanism(s) by which cotreatment of infected cells with IL-12+IL-18 restricts intracellular mycobacterial growth. Because cotreatment with IL-12+IL-18 similarly inhibited *M. tuberculosis* and BCG growth in host cells, we used BCG infection for the in-depth mechanistic manipulations in a biosafety level-2 laboratory, rather than those involving *M. tuberculosis* in complicated BSL3 conditions. We sought to determine which anti-*Mycobacterium* effector functions were activated by IL-12+IL-18 during infection of target cells. Because mycobacteria can activate the expression of antimicrobial defense peptides in lung epithelial cells and human macrophages (28–30), we rationalized that treatment with IL-12+IL-18 would enhance the expression of these peptides, resulting in growth inhibition of intracellular mycobacteria. To address this, BCG-infected cells were treated with IL-12+IL-18 together or individually or with media control, and RT-qPCR (see primers information in Table I) was used to assess the expression of *VDR* downstream genes *CAMP* and *DEFB4A*, encoding cathelicidin and β-defensin 2, respectively, antimicrobial peptides that are capable of killing intracellular mycobacteria (30, 31). These experiments revealed that cotreatment with IL-12+IL-18 significantly increased the expression of *CAMP* by up to ∼7-fold after BCG infection of A549 cells, THP-1 cells, and hMDMs (Fig. 3A). Similarly, IL-12+IL-18 cosignaling also increased *DEFB4A* expression by up to ∼8-fold (Fig. 3B). IL-12+IL-18 treatment still led to enhanced expression of CAMP and DEFB4A in H37Rv-infected hMDMs (Fig. 2C). This result is confirmed by the enhanced expression of protein levels of CAMP in IL-12+IL-18–treated

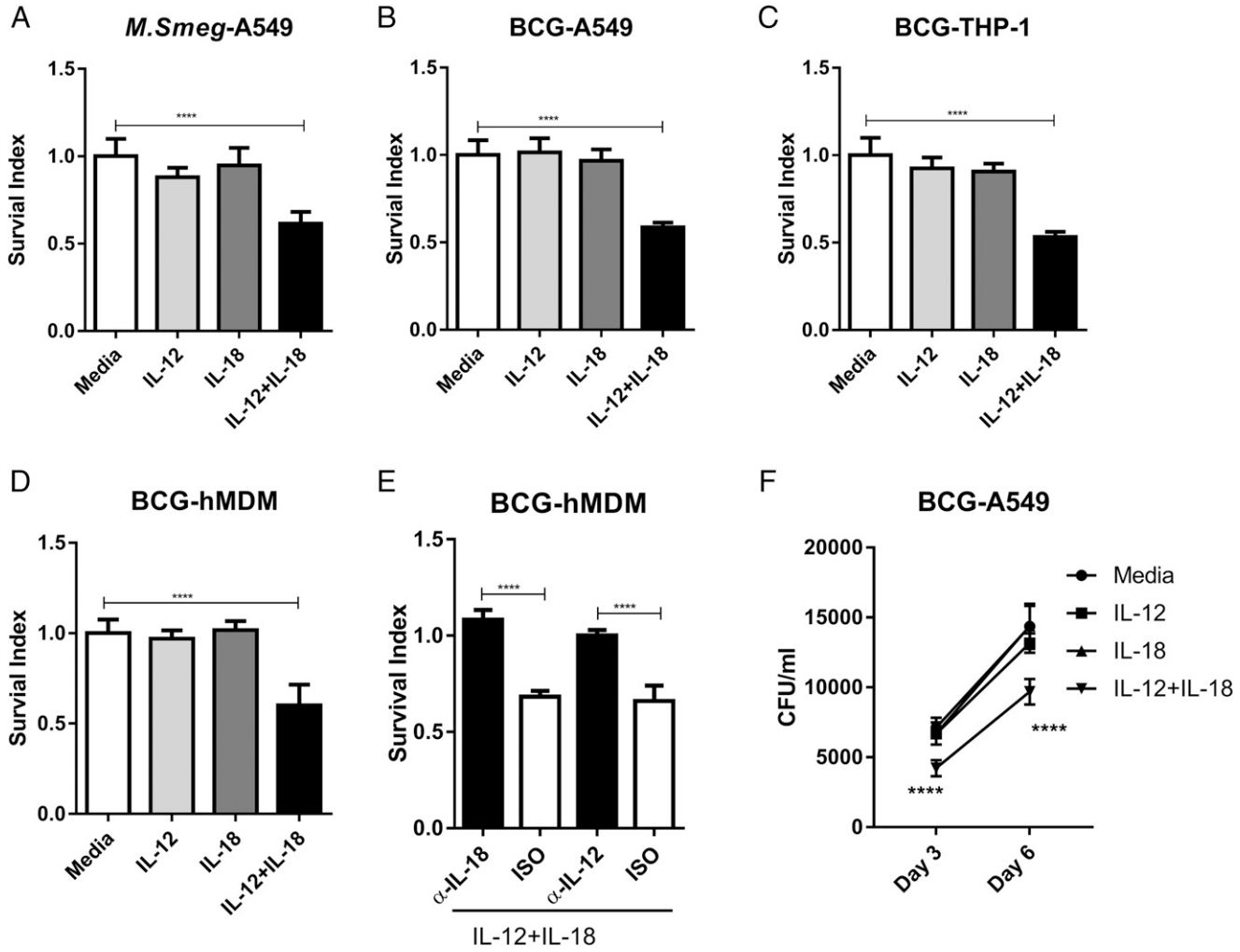

FIGURE 1. IL-12+IL-18 cotreatment of *Mycobacterium*-infected cells from monocytic and lung epithelial lineages led to reproducible inhibition of intracellular mycobacterial growth. (**A** and **B**) Survival index reflects the IL-12+IL-18–induced inhibition of intracellular growth of *M. smegmatis* or *M. bovis* BCG in A549 cells. A549 cells were infected with *M. smegmatis* or BCG and then cultured for 3 d with 50 ng/ml recombinant human IL-12, recombinant human IL-18, their combination, or culture media alone. (**C**) Mean survival index for BCG in THP-1 cells cultured with different treatments. THP-1 cells were first treated with 50 ng/ml PMA for differentiation. Then, differentiated THP-1 cells were infected with BCG and cultured for 3 d with 50 ng/ml recombinant human IL-12, recombinant human IL-18, their combination, or culture media alone. (**D** and **E**) Mean survival index for BCG in hMDMs treated as above. Adherent monocytes enriched from PBMCs were allowed to differentiate into human primary macrophages under stimulation (50 ng/ml M-CSF) in culture. hMDMs were infected with BCG and then cultured for 3 d with 50 ng/ml recombinant human IL-12, recombinant human IL-18, their combination, or culture media alone, in the absence or presence of Ab to IL-12 or IL-18 and isotype controls. (**F**) IL-12+IL-18 cosignaling decreased the number of intracellular BCG in A549 cells at days 3 and 6. A549 cells were infected with BCG and then cultured for 3–6 d with 50 ng/ml recombinant human IL-12, recombinant human IL-18, or their combination. Data in (A)–(C) are pooled from five independent experiments; data in (D) and (E) are pooled from four independent experiments using cells from 20 healthy donors; data in (F) are pooled from eight replicates in two independent experiments. ****$p < 0.0001$ versus media, ANOVA, followed by the Dunnett test (A–D); ANOVA, followed by the Tukey multiple-comparison test (E); versus media, repeated ANOVA, followed by the Dunnett test (F).

BCG-infected A549 and THP-1 cells, as measured by Western blot (Fig. 3C). Furthermore, when the expression of CAMP was impaired (Fig. 3D, 3E), IL-12+IL-18 treatment failed to control the growth of intracellular BCG compared with media (Fig. 3F, 3G). These results suggest that IL-12+IL-18 cosignaling led to enhanced expression of genes encoding antimycobacterial peptide, such as CAMP and DEFB4A, during BCG infection of monocytic and lung epithelial cell lineages, which contributed to the IL-12 +IL-18–mediated inhibitory effect on intracellular mycobacteria growth.

Because VDR signaling is required for the induction of *CAMP* and *DEFB4A*, and it mediates the control of intracellular pathogens (32, 33), we hypothesized that VDR activation of cathelicidin and β-defensin 2 might be required for the IL-12+IL-18–induced growth restriction of intracellular mycobacteria. Initially, we found that IL-12+IL-18 cosignaling significantly increased the expression of *VDR* by 6.4 ± 0.6–fold and 4.9 ± 0.87–fold during BCG infection of A549 cells and hMDMs, respectively, but not THP-1 cells (Fig. 4A). To prove that VDR activation of antimycobacterial peptides was required for the IL-12+IL-18 triggering of the antimycobacterial response, we developed stably transduced A549 and THP-1 cell lines displaying VDR knockdown (Fig. 4B, 4E). Notably, knockdown of VDR in BCG-infected shVDR-transduced A549 cells diminished the ability of cotreatment with IL-12+IL-18 to upregulate CAMP and DEFB4A genes compared with control shControl-transduced A549 cells (Fig. 4C). CAMP protein expression was also reduced by shRNA knockdown of VDR in BCG-infected A549 and THP-1 cells treated with IL-12+IL-18 (Fig. 4G). More importantly, shRNA knockdown of VDR in BCG-infected A549 and THP-1 cells

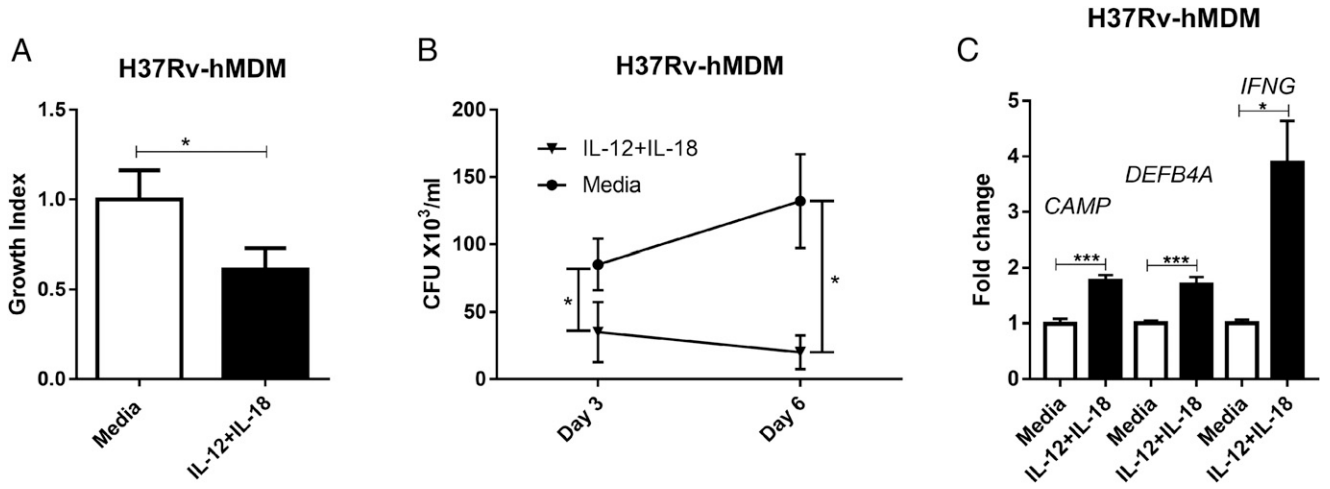

FIGURE 2. IL-12+IL-18 cotreatment in hMDMs consistently inhibited intracellular growth of virulent *M. tuberculosis* H37Rv. (**A**) Mean survival index for intracellular growth inhibition of *M. tuberculosis* H37Rv strain in hMDMs. *M. tuberculosis*–infected hMDMs were treated with IL-12+IL-18 or media for 3 d. Viable bacteria recovered from infected cells were quantified by counting CFU. Data are pooled from two independent experiments using cells from eight healthy uninfected donors. $*p < 0.05$, nonparametric *t* test. (**B**) IL-12+IL-18 cosignaling decreased the number of intracellular *M. tuberculosis* H37Rv bacilli in hMDMs at days 3 and 6. hMDMs were infected with H37Rv and then cultured for 3–6 d in the presence of 50 ng/ml recombinant human IL-12+IL-18 or media. Data are pooled from two independent experiments from eight uninfected donors. Individual cytokines alone were not found to inhibit the growth of BCG (Fig. 1) and were not evaluated. Attenuated BCG may be similarly inhibited as *M. tuberculosis* in IL-12+IL-18–treated hMDMs (Fig. 1D, 1E). Presumably, at day 6, BCG may be restricted similarly to *M. tuberculosis* in IL-12+IL-18–stimulated hMDMs. $*p < 0.05$, nonparametric *t* test. (**C**) Mean fold changes in the expression levels of *CAMP*, *DEFB4A*, and *IFNG* in H37Rv-infected hMDMs in the presence of media or IL-12+IL-18. Data are from two independent experiments using hMDMs from eight healthy uninfected donors. $*p < 0.05$, $***p < 0.001$, *t* test.

abrogated the ability of IL-12+IL-18 cotreatment to restrict the growth of intracellular mycobacteria (Fig. 4D, 4F). Taken together, these data suggest that VDR activation is required for the IL-12+IL-18–induced restriction of intracellular mycobacterial growth in macrophages and lung epithelial cells.

The p38–MAPK and STAT4 pathways are involved in IL-12+IL-18 cosignaling that led to inhibition of intracellular mycobacteria

Next, studies of T cells and NK cells showed that IL-18 could activate NF-κB and MAPK–associated PI3K, MEK, and p38 signaling pathways (34, 35). Therefore, we investigated which of these pathways in monocytes was required for the IL-12+IL-18 cosignaling that induced the growth inhibition of intracellular mycobacteria. Thus, BCG-infected A549 and hMDM cells were incubated with individual well-documented inhibitors of the MAPK and NF-κB pathways in the presence of IL-12+IL-18. Notably, inhibitors of the NF-κB pathway (PDTC) and the MEK-MAPK pathway (U0126-EtOH) did not appear to alter the IL-12+IL-18–induced restriction of intracellular BCG growth (Fig. 5A, 5B). In contrast, the p38–MAPK inhibitor (SB203580) significantly blocked the IL-12+IL-18 cosignaling–induced growth restriction of mycobacteria in BCG-infected hMDMs and A549 cells (Fig. 5A, 5B). Consistently, we found that IL-12+IL-18 cosignaling could modulate the expression of IL-18Rs in hMDMs, as seen in T cells and NK cells (15, 34, 35), because cotreatment with IL-12+IL-18 significantly increased the expression of *IL-18R1* and *IL-18RAP*, by 5.7 ± 2.1 and 5.2 ± 2.6–fold, respectively, in mycobacteria-infected hMDMs (Fig. 5C). Furthermore, treatment with the p38–MAPK inhibitor (SB203580) significantly inhibited IL-12+IL-18 cosignaling–induced CAMP at the mRNA and protein levels in BCG-infected hMDMs and THP-1 cells (Fig. 5D, 5E). These results suggest that the combined action of IL-12+IL-18 that resulted in the growth inhibition of intracellular mycobacteria mainly occurred via the p38–MAPK signaling axis and not via the NF-κB or MEK–MAPK pathway.

Because studies of T cells and NK cells identified STAT4 as a major downstream molecule for IL-12 signaling (36), we presumed that the IL-12/STAT4 pathway in monocytic/lung epithelial lineages might contribute to IL-12+IL-18 treatment resulting in the growth inhibition of intracellular mycobacteria. To test this, we produced lentivirus shRNA-knockdown constructs and developed stable A549 and THP-1 cell lines displaying STAT4 knockdown, with shControl serving as control. STAT4 knockdown was confirmed in these transduced A549 and THP-1 cells (Fig. 5F, 5H). Clearly, knocking down STAT4 expression in A549 and THP-1 cells reversed the IL-12+IL-18–induced growth inhibition of intracellular mycobacteria (Fig. 5G, 5I). Notably, the reversing effect by the STAT4 knockdown correlated with the reduced

Table I. Primer sequences for RT-qPCR or RT-PCR used in this study

Gene Symbol	Forward (5′–3′)	Reverse (5′–3′)
DEFB4A	ATCAGCCATGAGGGTCTTGT	GAGACCACAGGTGCCAATTT
CAMP	AGGATTGTGACTTCAAGAAGGACG	GTTTATTTCTCAGAGCCCAGAAGC
IL-18R1	GTTGAGTTGAATGACACAGG	TCCACTGCAACATGGTTAAG
IL-18Rbeta	CTGGACAGAACTCACAGCTC	TCAAAGGCTCTAAACCACAG
STAT4	CAGTGAAAGCCATCTCGGAGGA	TGTAGTCTCGCAGGATGTCAGC
IFNGR1	CATCACGTCATACCAGCCATTT	CATCACGTCATACCAGCCATTT
IFNGR2	CTCCATTCTGCCTGGGTGACAA	CGTGGAGGTATCAGCGATGTCA
VDR	CTGACCCTGGAGACTTTGAC	TTCCTCTGCACTTCCTCATC
EF1A	GATTACAGGGACATCTCAGGCTG	TATCTCTTCTGGCTGTAGGGTGG

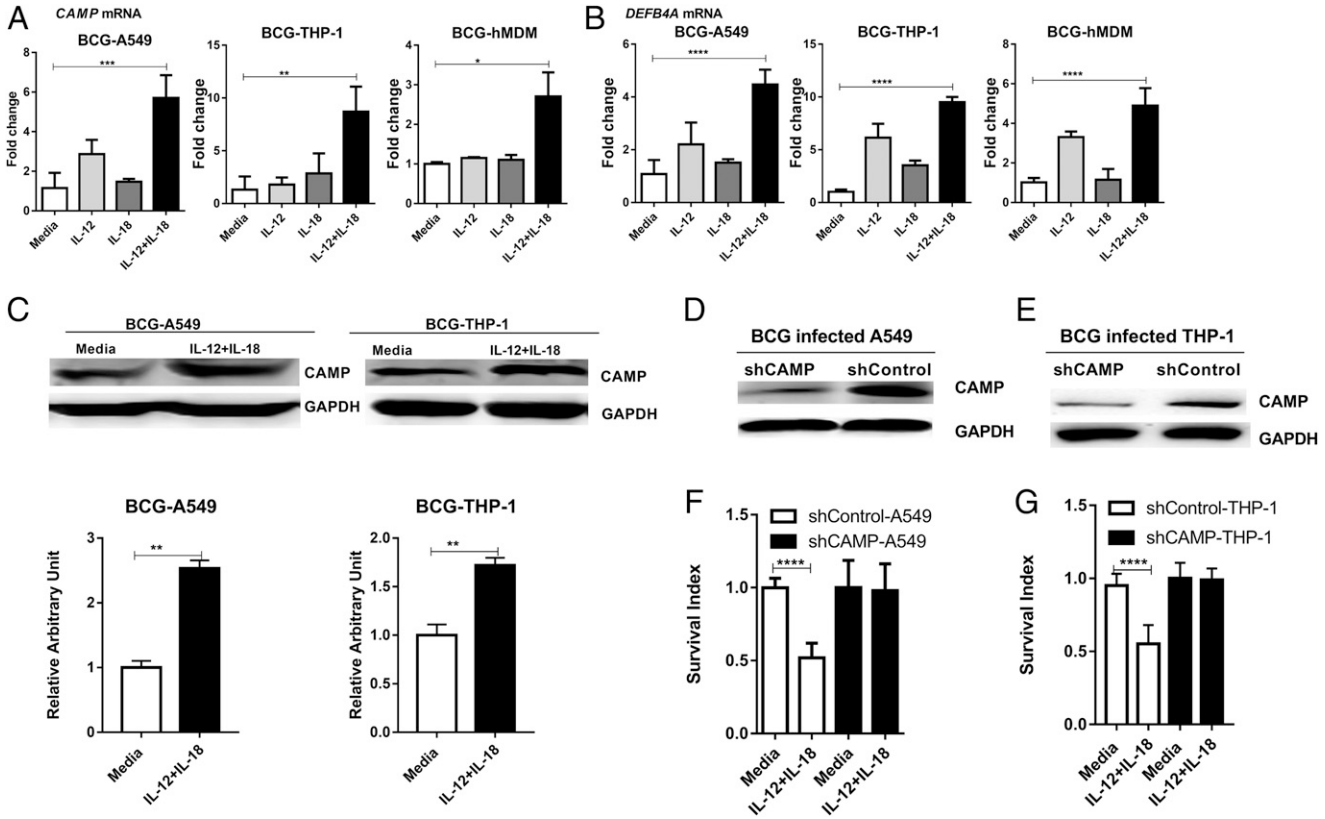

FIGURE 3. CAMP appeared to be required for the IL-12+IL-18–mediated growth inhibition of intracellular mycobacteria. Mean fold changes in the expression levels of *CAMP* (**A**) and *DEFB4A* (**B**) in BCG-infected A549 cells, THP-1 cells, and hMDMs in the presence of media, IL-12, IL-18, or IL-12+IL-18 for 24 h. Data are from four independent experiments using A549 or THP-1 cells and three independent experiments using hMDMs from 12 healthy uninfected donors. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ versus media, ANOVA, followed by the Dunnett test. (**C**) IL-12+IL-18 cosignaling led to enhanced expression of CAMP protein levels in BCG-infected A549 cells (left panels) and BCG-infected THP-1 cells (right panels). Representative Western blots from four replicates (upper panels) and quantitative analysis of CAMP protein levels by densitometry (lower panels). $**p < 0.01$, *t* test. (**D** and **E**) Representative Western blots demonstrating shRNA knockdown of CAMP in A549 and THP-1 cells compared with the shRNA-Control. (**F** and **G**) Bar graphs showing that shRNA knockdown of CAMP reverses the growth restriction of intracellular mycobacteria during IL-12+IL-18 treatment of BCG-infected A549 and THP-1 cells compared with shRNA control. Data are derived from eight replicates in two independent experiments. $****p < 0.001$, *t* test.

ability of IL-12+IL-18 to upregulate CAMP protein compared with the control (Fig. 5J). Taken together, these data suggest that the p38–MAPK and STAT4 pathways are required for the combined activity of IL-12+IL-18 in the restriction of intracellular mycobacterial growth in monocytes and pulmonary epithelial cells.

Autophagy, but not caspase-mediated apoptosis, was involved in IL-12+IL-18 cosignaling inhibition of intracellular mycobacteria growth

VDR activation of antimicrobial peptides has been shown to induce autophagy and mycobactericidal activity (37). Thus, we sought to examine whether autophagy was involved in the cosignaling induced by IL-12+IL-18 that triggered restriction of intracellular mycobacterial growth. We assessed an increase in the LC3II/LC3I ratio or LC3II conversion as a parameter to examine autophagic flux in BCG-infected target cells treated with IL-12+IL-18 or control. We found that the IL-12+IL-18 cosignaling in BCG-infected A549 cells led to an increase in the LC3II/LC3I ratio (i.e., autophagy flux) (Fig. 6A) beyond the background autophagy induced by BCG infection of A549 cells in the absence of cytokines (38, 39). In parallel, we developed a stable THP-1 cell line expressing GFP-LC3 using a lentiviral vector to support our results from Western blot assays. Consistently, IL-12+IL-18 treatment remarkably formed fluorescent LC3 aggregates/microdots in GFP-LC3–transduced BCG-infected THP-1 cells (Fig. 6B). Because p38, STAT4, and VDR were indispensable for the IL-12+IL-18–induced antimicrobial response, we sought to determine whether autophagy was involved in connecting p38/STAT4/VDR activation and growth inhibition of intracellular mycobacteria. To this end, we examined whether the conversion of LC3I to LC3II was altered by p38 inhibition, STAT4 knockdown, VDR knockdown, or CAMP knockdown during IL-12+IL-18 treatment of BCG-infected THP-1 cells. Treatment with the p38 inhibitor SB203580 reduced IL-12+IL-18–induced LC3II conversion compared with the control (DMSO) (Fig. 6C). This was consistent with the decreased formation of LC3II by two well-documented autophagy inhibitors: wortmannin and 3-MA (40, 41) (Fig. 6C). Moreover, knockdown of CAMP by shRNA significantly reduced the conversion of LC3I to LC3II during IL-12+IL-18 treatment of BCG-infected THP-1 cells (Fig. 6D), suggesting a critical role for CAMP in the IL-12+IL-18 activation of autophagy and BCG growth inhibition in THP-1 cells. Because we already showed that CAMP was upregulated upstream by VDR (Fig. 4G) and STAT4 (Fig. 5J), we sought to investigate whether shRNA knockdown of VDR or STAT4 would impact autophagy. As expected, knocking down VDR or STAT4 reduced the formation of LC3II during the IL-12+IL-18 treatment of BCG-infected THP-1 cells (Fig. 6E). These

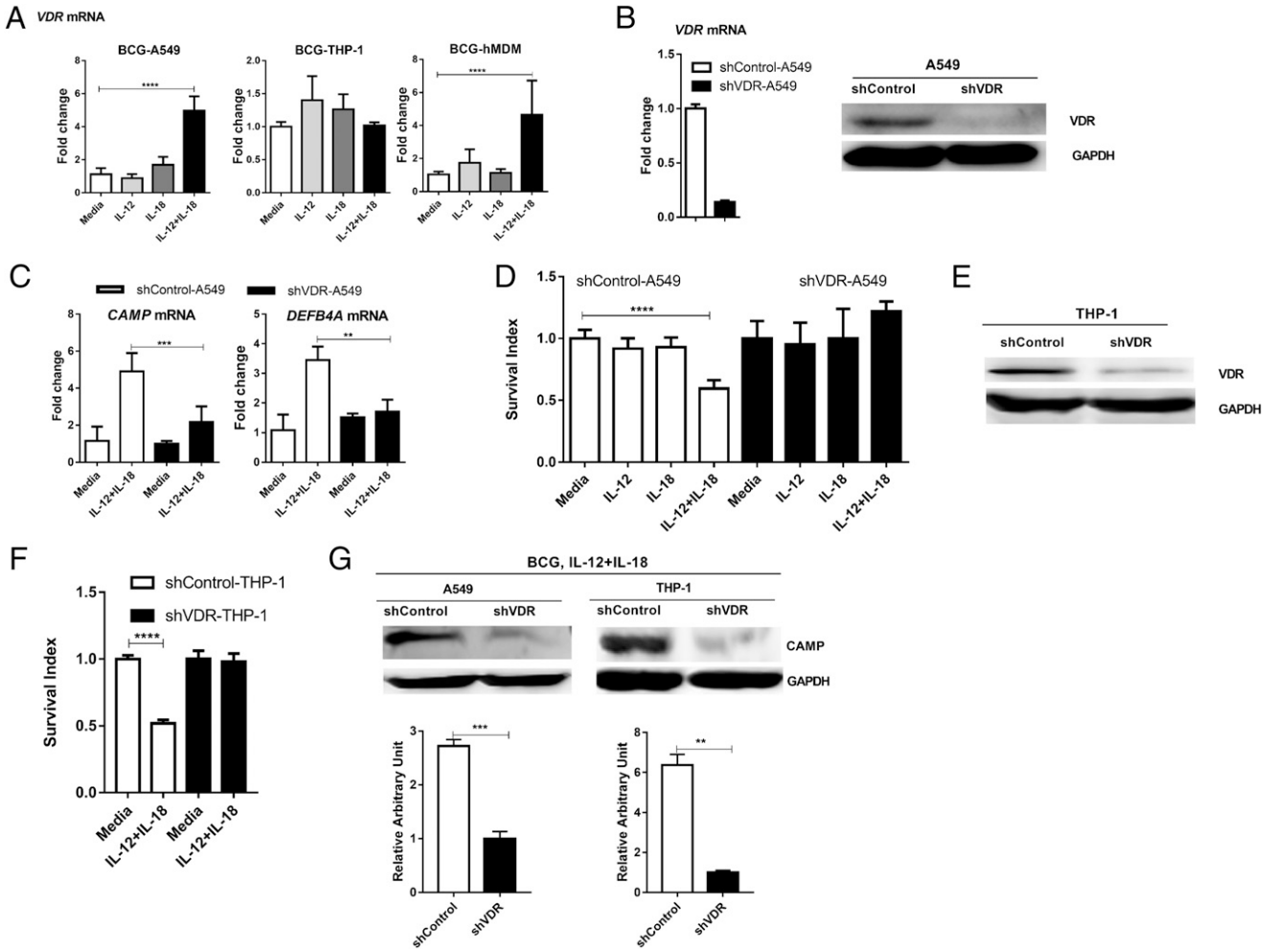

FIGURE 4. VDR activation of antimicrobial peptides appeared to be required for the IL-12+IL-18–mediated growth inhibition of intracellular mycobacteria. (**A**) Mean fold changes in the expression levels of *VDR* in BCG-infected A549 cells, THP-1 cells, and hMDMs in the presence of media, IL-12, IL-18, or IL-12+IL-18. Data are from four independent experiments using A549 or THP-1 cells and from three independent experiments using hMDMs from 12 healthy uninfected donors. $****p < 0.0001$ versus media, ANOVA, followed by the Dunnett test. (**B**) Representative data demonstrating knockdown of VDR at the mRNA level (left panel, RT-qPCR) and at the protein level (right panel, Western blot) in A549 cells stably transduced with the lentivirus construct LV-shVDR in comparison with the control. Stably transduced cell lines were selected by puromycin (2 μg/ml) treatment of cultures. (**C**) Fold changes in the expression levels of *CAMP* and *DEFB4A* in BCG-infected shVDR- and shControl-transduced A549 cells in the presence of media or IL-12 +IL-18 for 24 h. Data were generated by RT-qPCR and pooled from three independent experiments. $**p < 0.01$, $***p < 0.001$, *t* test. (**D**) Changes in mean survival index for BCG in BCG-infected shVDR- and shControl-transduced A549 cells in the presence of media, IL-12, IL-18, or IL-12+IL-18. Data are derived from four independent experiments. Note that VDR knockdown led to reversion of IL-12+IL-18–mediated inhibition of BCG growth. $****p <$ 0.0001 versus media, ANOVA, followed by the Dunnett test. (**E**) Representative Western blot demonstrating VDR knockdown in THP-1 cells in comparison with the shRNA control. (**F**) Bar graph shows that shRNA knockdown of VDR reverses the growth restriction of intracellular mycobacteria during IL-12 +IL-18 treatment of BCG-infected THP-1 cells compared with shRNA control. Data are derived from eight replicates in two independent experiments. $****p < 0.0001$, *t* test. (**G**) Representative Western blots (from four replicates) show that shRNA knockdown of VDR abrogated the ability of IL-12+IL-18 treatment to induce CAMP expression in BCG-infected A549 and THP-1 cells (upper panel). The bar graphs show quantitative analysis of CAMP protein levels by densitometry (lower panels). $**p < 0.01$, $***p < 0.001$, *t* test.

results suggest that the IL-12+IL-18–mediated activation of p38/STAT4 can activate VDR-derived CAMP and the autophagy process.

We then questioned whether the autophagy induced by IL-12 +IL-18 cosignaling contributed to the detectable inhibition of intracellular mycobacterial growth. To address this, wortmannin and 3-MA were used for blocking autophagy flux in the context of IL-12+IL-18–mediated growth restriction of intracellular mycobacteria. The apoptosis inhibitor zVAD.fmk was evaluated in parallel, because it inhibits caspase-mediated apoptosis (42). Notably, the addition of 3-MA or wortmannin to BCG-infected A549 and THP-1 cultures significantly blocked the IL-12+IL-18 cosignaling restriction of intracellular mycobacterial growth compared with DMSO control (Fig. 6F). In contrast, the apoptosis inhibitor zVAD.fmk did not have significant blocking activity in BCG-infected A549 cells and hMDMs (Fig. 6G). Taken together, these data indicate that the IL-12+IL-18–mediated activation of p38 and STAT4 activates CAMP and autophagy and leads to the growth inhibition of intracellular mycobacteria.

Activation of the IFN-γ–IL-32 pathway was required for IL-12 +IL-18 cosignaling–mediated growth inhibition of intracellular mycobacteria

Finally, we sought to explore the possibility that the combined action of IL-12+IL-18 activates IFN-γ and/or TNF-α, resulting

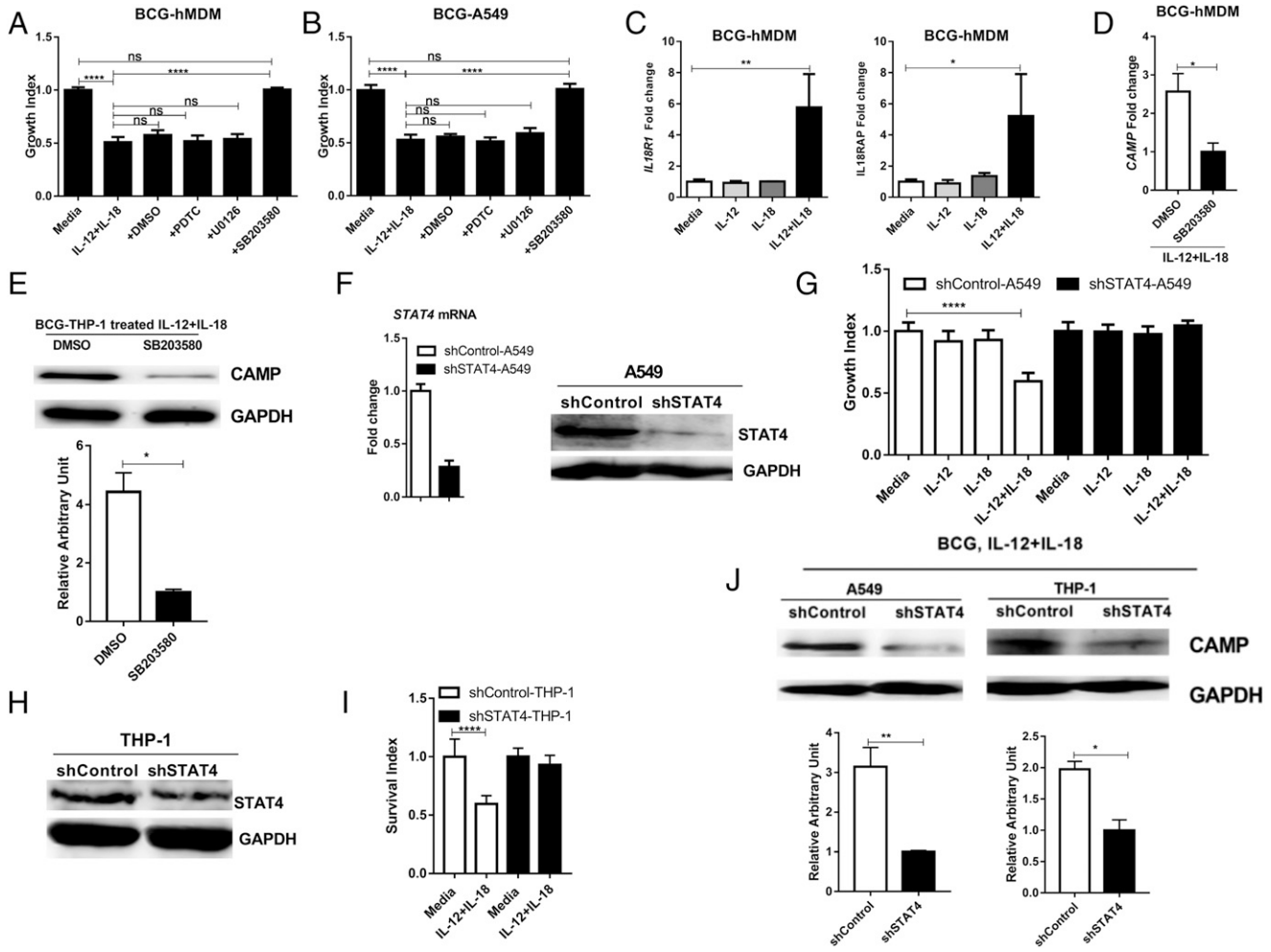

FIGURE 5. p38–MAPK and STAT4 pathways were indispensable to IL-12+IL-18–mediated inhibition of intracellular mycobacteria. Changes in the mean survival index of BCG in IL-12+IL-18–cotreated A549 cells (**A**) and hMDMs (**B**) in the absence and presence of the chemical inhibitors PDTC (10 μM), U0126 (10 μM), SB203580 (10 μM), and DMSO in the 3-d culture. Data were pooled from three independent experiments using A549 cells (A) and five experiments using hMDMs derived from 15 healthy uninfected donors (B). $^{****}p < 0.0001$, ANOVA, followed by the Tukey multiple-comparisons test. (**C**) Mean fold changes in the expression levels of the IL-18R genes *IL-18R1* and *IL-18RAP* in BCG-infected hMDMs incubated for 24 h with media, IL-12, IL-18, or IL-12+IL-18. Fold changes were calculated using the expression levels of cytokine treatment versus those of media control. Data were generated using RT-qPCR and pooled from three independent experiments using hMDMs from 12 healthy uninfected donors. $^{*}p < 0.05$, $^{**}p < 0.01$ versus media, ANOVA, followed by the Dunnett test. Inhibition of p38 hindered enhanced expression of *CAMP* mRNA levels in BCG-infected hMDMs (**D**) and CAMP protein levels in BCG-infected THP-1 cells (**E**) treated by IL-12+IL-18. Data in (D) are derived from three independent assays using nine healthy uninfected donors. (E) The Western blot is a representative from four replicates (upper panel). The bar graph shows pooled data for OD analysis (lower panel). $^{*}p < 0.05$, *t* test. (**F**) Representative data demonstrating knockdown of the transcription factor STAT4 in A549 cells stably transduced with the lentiviral construct LV-shSTAT4 in comparison with the control. (**G**) Mean survival index for BCG in shSTAT4- and shControl-transduced A549 cells in the presence of media, IL-12, IL-18, or IL-12+IL-18. Note the reversion of IL-12+IL-18–mediated inhibition in the setting of STAT4 knockdown. Data were derived from four independent experiments. $^{****}p < 0.0001$ versus media, ANOVA, followed by the Dunnett test. (**H**) Representative Western blot shows shRNA knockdown of STAT4 in THP-1 cells in comparison with the shRNA control. (**I**) Bar graph shows that shRNA knockdown of STAT4 reverses the growth restriction of intracellular mycobacteria during IL-12+IL-18 treatment of BCG-infected THP-1 cells compared with the shRNA control. Data are derived from eight replicates in two independent experiments. $^{****}p < 0.001$, *t* test. (**J**) Representative Western blots (from four replicates) shows that shRNA knockdown of STAT4 reduced CAMP protein expression levels during IL-12+IL-18 treatment of BCG-infected A549 and THP-1 cells (upper panel). The bar graphs show quantitative analysis of CAMP protein levels by densitometry (lower panels). $^{*}p < 0.05$, $^{**}p < 0.01$, *t* test. ns, not significant.

in the induction of downstream genes that mediate growth inhibition of intracellular mycobacteria (32, 41, 43–45). hMDMs, infected or not with BCG, were treated with media, IL-12, IL-18, or IL-12+IL-18 for 3 d, and culture supernatants were assessed for IFN-γ and TNF-α. Treatment of hMDMs by IL-12 and/or IL-18 without BCG infection did not significantly induce secretion of IFN-γ and TNF-α in culture supernatant (data not shown). Similarly, in BCG-infected hMDMs, IL-18 alone did not induce IFN-γ release; however, IL-12 did so to a moderate degree in BCG-infected macrophages. Cotreatment of BCG-infected hMDMs with IL-12+IL-18 led to secretion of significantly higher levels of IFN-γ than did the addition of IL-12 alone (Fig. 7A, 444 ± 15 versus 275 ± 21 pg/ml), suggesting that additional innate signaling from mycobacteria was required for induction of IFN-γ. In contrast, there was no significant change in TNF-α secretion (Fig. 7B). Consistently, cotreatment with IL-12+IL-18 upregulated expression of the IFN-γ receptors IFNGR1 and IFNGR2 on target cells at the mRNA level (Supplemental Fig. 3C). In addition, IL-12+IL-18, although facilitating

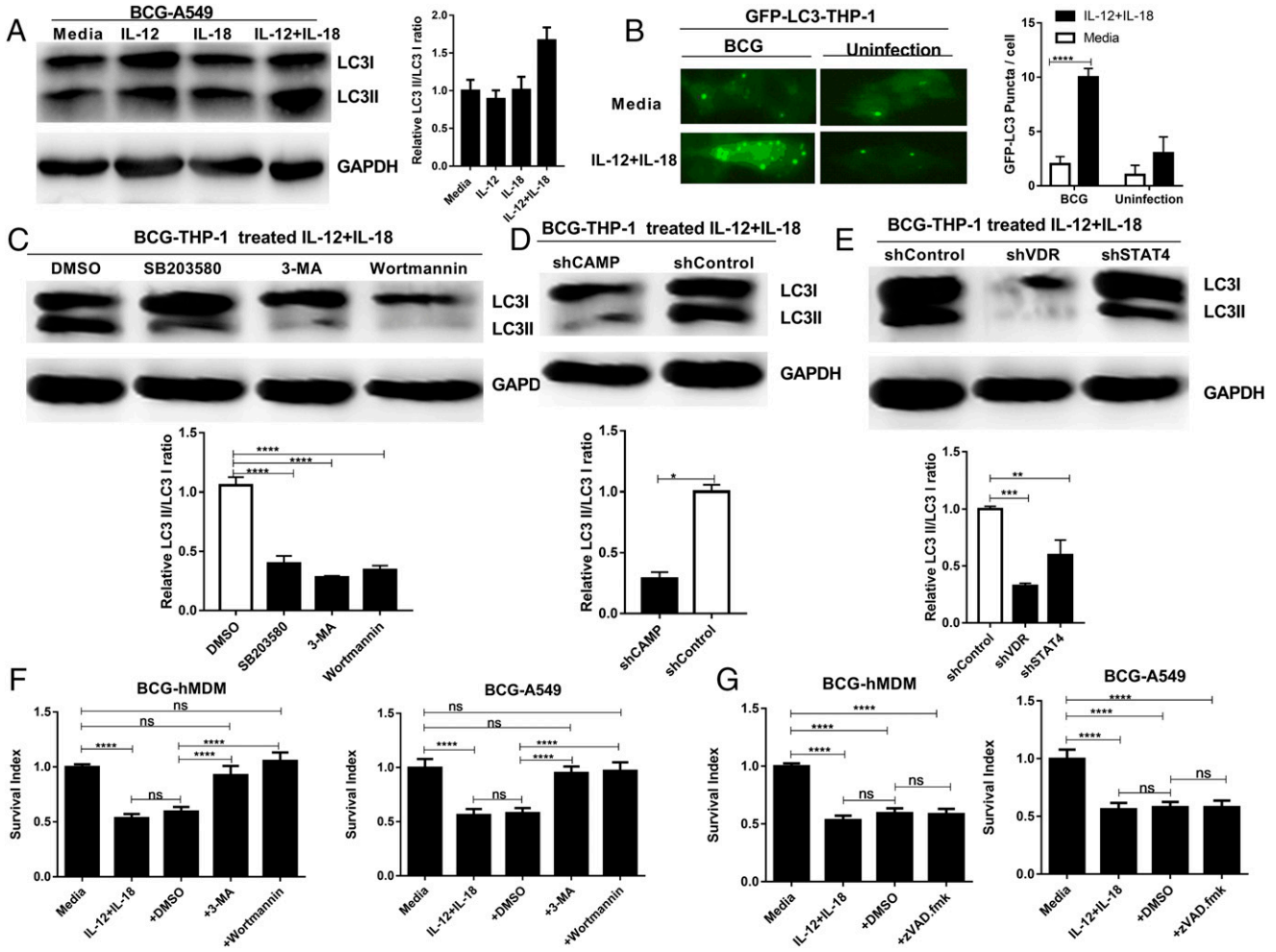

FIGURE 6. Autophagy, but not caspase-mediated apoptosis, was involved in the IL-12+IL-18–induced inhibition of intracellular mycobacteria growth. (**A**) Representative Western blots (left panel) displaying autophagy-related proteins LC3II and LC3I in lysates from BCG-infected hMDMs treated for 6 h with media, IL-12, IL-18, or IL-12+IL-18. Bar graph of the ratio of the intensities of LC3II/LC3I bands from the Western blot (right panel, $n = 3$). (**B**) Representative fluorescence images of individual cells treated with IL-12+IL-18 or media control in the presence or absence of BCG infection showing that GFP-LC3 punctae or fluorescent GFP-LC3 aggregates of the autophagy machinery were formed after IL-12+IL-18 treatment of GFP-LC3–transduced BCG-infected THP-1 cells (left panel; original magnification ×400). Bar graph showing comparative quantitation of GFP-LC3 punctae per cell (right panel; $n \geq 25$ cells for each group in three independent experiments). Low background of fluorescence dots is seen in GFP-LC3 THP-1 cells treated with media or IL-12+IL-18. Representative Western blots show that conversion of LC3II (autophagy flux) was reduced by the p38 inhibitor SB203580/autophagy inhibitors 3-MA and wortmannin (**C**) or shRNA knockdown of CAMP, VDR, and STAT4 (**D** and **E**) during IL-12+IL-18 treatment of BCG-infected THP-1 cells from four replicates (upper panels). Quantitative analysis of relative LC3II/LC3I ratios by densitometry (lower panels). $^{****}p < 0.0001$ versus DMSO, ANOVA followed by the Dunnett test (C). $^{*}p < 0.05$, t test (D); $^{**}p < 0.01$, $^{***}p < 0.001$ versus shControl, ANOVA, followed by the Dunnett test (E). (**F**) A 3-d treatment with the autophagy-blocking reagent 3-MA (10 μM) or wortmannin (10 μM) could abrogate the IL-12+IL-18–mediated inhibition of intracellular BCG in BCG-infected hMDM or A549 cells compared with DMSO control. (**G**) A 3-d treatment with the apoptosis-blocking reagent zVAD.fmk (10 μM) was not able to abrogate the IL-12+IL-18–mediated inhibition of intracellular BCG in BCG-infected hMDMs or A549 cells. In the absence of IL-12+IL-18, the autophagy inhibitors 3-MA and wortmannin, but not zVAD.fmk, have reversed impact on mycobacterial growth (Supplemental Fig. 3B). Data in (F) and (G) are pooled from four independent experiments using A549 cells and three independent experiments using hMDMs from 12 healthy uninfected donors. $^{****}p < 0.0001$, ANOVA, followed by the Tukey multiple-comparisons test. ns, not significant.

IFN-γ production, led to the induction of significantly higher levels of IL-32 than did treatment with IL-18 alone (Fig. 7C), implicating a possible IL-12+IL-18 → IFN-γ → IL-32 activation axis (32, 43).

To authenticate that IL-12+IL-18 activation of the IFN-γ pathway contributed to inhibition of intracellular mycobacteria, we blocked IFN-γ or TNF-α with neutralizing mAbs. The addition of neutralizing anti–IFN-γ Ab, but not anti–TNF-α Ab, significantly abrogated the IL-12+IL-18–mediated growth inhibition of intracellular mycobacteria in BCG-infected hMDMs (Fig. 7D). Consistently, blockade of IFN-γ also significantly reduced the IL-12+IL-18–mediated induction of IL-15 and IL-32 (Fig. 7E). Moreover, neutralization of IFN-γ in IL-12+IL-18–treated BCG-infected hMDMs resulted in reduced expression of CAMP and decreased autophagy (Fig. 7F, 7G). These data suggested that activation of the IL-12+IL-18 → IFN-γ → IL-15/IL-32 → CAMP → autophagy pathway was required for the IL-12+IL-18–induced growth inhibition of intracellular mycobacteria. Of note, IL-12 +IL-18 activation of the IL-12+IL-18 → IFN-γ → IL-15 → IL-32 pathway did not appear to be negated by type I IFN (IFN-β), which was previously shown to inhibit IFN-γ–induced antimicrobial capability (46, 47). The addition of IFN-β enhanced

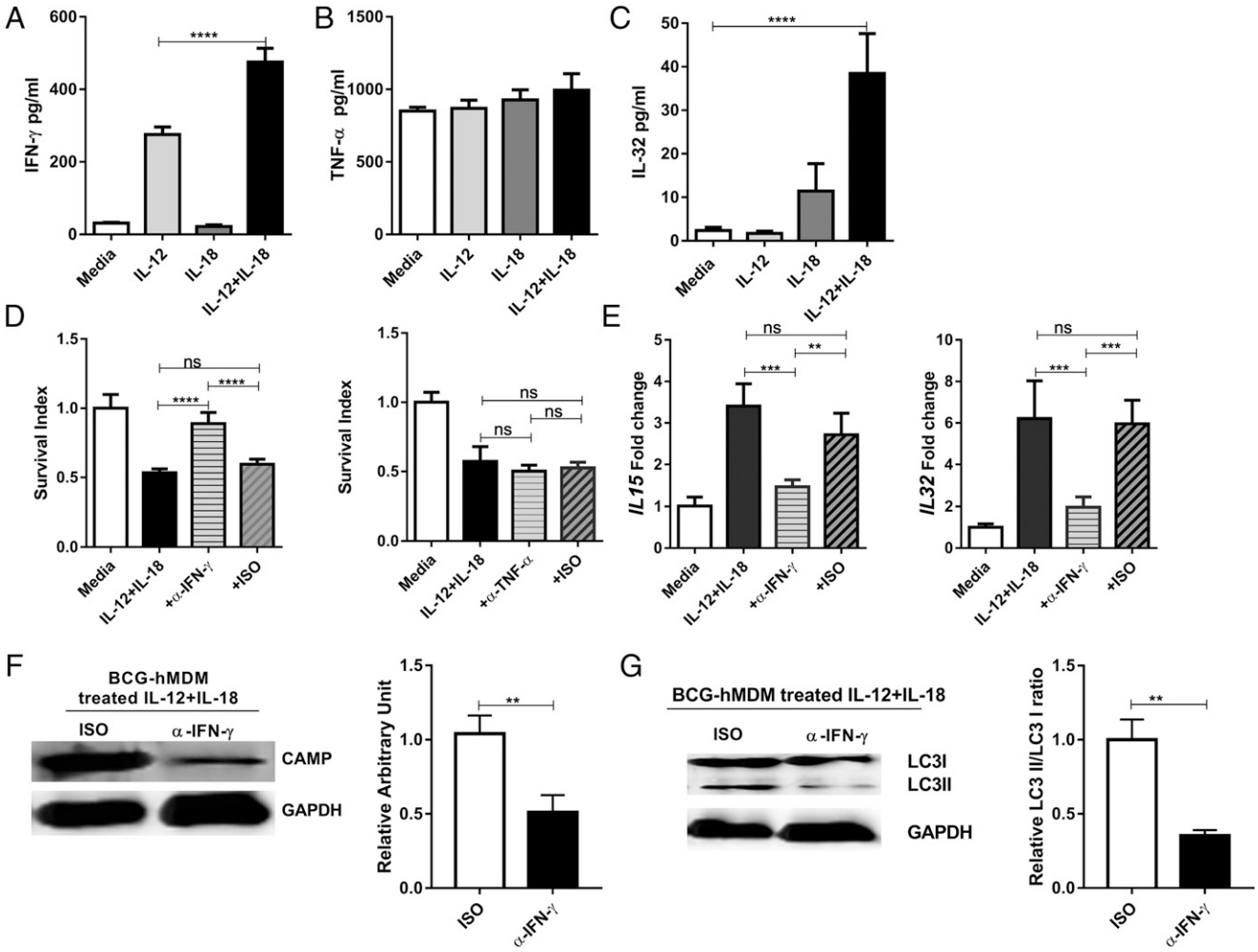

FIGURE 7. Activation of the IFN-γ–dependent antimycobacterial pathway was required for the IL-12+IL-18 cosignaling–mediated growth inhibition of intracellular mycobacteria in human macrophages. Mean cytokine protein concentrations for IFN-γ (**A**), TNF-α (**B**), and IL-32 (**C**) in culture supernatants from BCG-infected hMDMs in the presence of media, IL-12, IL-18, or IL-12+IL-18. Culture conditions were the same as in Fig. 1D. Cytokine proteins were measured by ELISA. Data were pooled from three independent experiments using hMDMs from 12 healthy uninfected donors. $^{****}p < 0.0001$, ANOVA, followed by the Tukey multiple-comparisons test. (**D**) IFN-γ blockade (left panel) but not TNF-α blockade (right panel) impacts the mean survival index for BCG in IL-12+IL-18–treated hMDMs. BCG-infected hMDMs were treated with media or IL-12+IL-18 in the presence of neutralizing anti–IFN-γ Ab, anti–TNF-α Ab, and their isotype controls (ISO) (5 μg/ml for each), CFU were counted, and the survival index was calculated. In the absence of IL-12 +IL-18, anti–IFN-γ or anti–TNF-α Ab does not lead to changes in intracellular mycobacterial growth (Supplemental Fig. 3A). Data are derived from three independent experiments using hMDMs from 12 healthy uninfected donors. $^{****}p < 0.0001$, ANOVA, followed by the Tukey multiple-comparisons test. (**E**) IFN-γ blockade–induced abrogation of intracellular BCG growth coincided with decreased expression of IL-15 and IL-32 in BCG-infected hMDMs in comparison with IL-12+IL-18 without anti–IFN-γ or ISO. The expression levels of *IL-15* and *IL-32* mRNA in BCG-infected hMDMs were quantified by RT-qPCR. Data are derived from three independent experiments using hMDMs from 12 healthy uninfected donors. $^{***}p < 0.001$, $^{**}p < 0.01$, ANOVA, followed by the Tukey multiple-comparisons test. (**F**) Representative Western blot shows that anti–IFN-γ–neutralizing Ab, but not ISO, reduced CAMP expression during IL-12+IL-18 treatment of BCG-infected hMDMs (left panel). Quantitative analysis of CAMP protein levels by densitometry (right panel). Data are derived from six replicates. $^{**}p < 0.01$, *t* test. (**G**) Representative Western blot shows that anti–IFN-γ–neutralizing Ab, but not ISO, reduced the conversion of LC3II during IL-12+IL-18 treatment of BCG-infected hMDMs (left panel). Quantitative analysis of relative LC3II/LC3I ratios by densitometry (right panel). Data are derived from six replicates. $^{**}p < 0.01$, *t* test. ns, not significant.

intracellular BCG growth in infected hMDMs in the absence of IL-12+IL-18 treatment but not in IL-12+IL-18–cotreated cells (Supplemental Fig. 4).

Discussion

Activation of the innate immune response triggers the release of cytokines that induce inflammation and instruct the adaptive T cell response. Two key innate cytokines induced by mycobacteria and involved in host defense are IL-12 (10–13, 16) and IL-18 (14–16, 48, 49). These cytokines act individually on NK and T cells (11, 17, 48, 50, 51); however, together, IL-12 and IL-18 provide a potent stimulus for innate instruction of the adaptive T cell response toward Th1 cell differentiation (17). The current study provides evidence that together, IL-12 and IL-18 trigger antimycobacterial responses against intracellular pathogens in innate cells, including macrophages and pulmonary epithelial cells, involving the cosignaling of p38–MAPK and STAT4, activation of VDR-derived CAMP or β-defensin 2, and autophagy. Moreover, the ability of IL-12+IL-18 to induce the production of IFN-γ by macrophages was required for upregulation of the antimicrobial activity against *M. tuberculosis*, identifying a common link between the innate and adaptive immune responses. Our data provide new insight into the combined role of IL-12+IL-18 in host defense, with clinical relevance in the context that polymorphisms

of IL-12 and IL-18 are reported in patients with tuberculosis (TB) (52, 53).

The ability of macrophages to upregulate antimicrobial peptide expression provides one mechanism of innate host defense against intracellular pathogens (30). BCG infection also triggers the activation of microbicidal peptides in the A549 epithelial cell line through multiple mechanisms (28, 29). Our data suggest that IL-12+IL-18 treatment induces the upregulation of the antimicrobial peptide genes *CAMP* and *DEFB4A*. Particularly, the increased patterns of CAMP and autophagy were observed in lung epithelial cells and human macrophages, suggesting a common mechanism of the IL-12+IL-18–induced antimicrobial response in these two different cell types. Consistent with multiple studies, the increased expression of antimicrobial peptide genes might represent the downstream effector function of the IL-12+IL-18–induced growth inhibition of intracellular mycobacteria (33, 54, 55). Consistent with the demonstration that both of these antimicrobial peptide genes are regulated by a transcription factor, VDR (31, 37, 55), we found that blockade of VDR signaling by transduction with shRNA lentivirus affected IL-12+IL-18–induced microbicidal activity. Furthermore, the requirement of VDR explains, in part, the vital role of autophagy in the clearance of intracellular bacteria induced by the combined action of IL-12+IL-18 (56).

IL-12 and IL-18 cooperate to activate cosignaling pathways and inhibit the intracellular growth of mycobacteria in target host cells, because the antimycobacterial effect cannot be induced by either alone or in the presence of anti–IL-12– or anti–IL-18–neutralizing Ab in the cotreatment culture. Together, IL-12+IL-18 leads to signaling via the p38–MAPK and STAT4 pathways, which are required to inhibit the growth of intracellular mycobacteria, because small molecule inhibition of p38–MAPK or knockdown of STAT4 clearly reduced the antimicrobial effect. Although blocking the p38–MAPK pathway strongly reduced the IL-12+IL-18–induced antimycobacterial activity, blocking the MEK–MAPK and NF-κB pathways had no effect. The discrepant requirement of specific MAPK pathways provides new insights into how the host intracellular pathway–network regulates intracellular pathogen survival (37).

The IL-12+IL-18–induced growth restriction of intracellular bacteria is dependent on VDR-derived CAMP and autophagy. IL-12+IL-18 upregulation of VDR mRNA in THP-1 cells is not as striking as that in A549 cells or hMDMs, presumably due to complex cell-type factors. However, knockdown of VDR protein by shRNA manipulation consistently abrogates the IL-12+IL-18–induced activation of antimicrobial peptides and reversed the IL-12+IL-18–mediated inhibition of BCG growth in THP-1 and A549 cells. These results suggest that VDR is required for the IL-12+IL-18–induced activation of a downstream antimicrobial pathway in both cell types. Autophagy has been shown to facilitate the delivery of antimicrobial peptides to the intracellular compartments containing the pathogen (57). The data in our study, using chemical inhibitors, emphasize the significant role of autophagy, which is consistent with multiple reports (32, 39, 58–60). Although caspase-mediated apoptosis has been associated with the suppression of intracellular bacterial growth (37, 61, 62), it has been suggested by other investigators that apoptosis may facilitate the spread of mycobacteria during infection in vivo in zebrafish and in a mouse model (63, 64). Our data suggest that IL-12+IL-18–induced antimycobacterial activity does not require the induction of apoptosis in BCG-infected hMDMs and A549 cells, indicating that autophagy, but not apoptosis, contributes to lower bacillary load. Also, these data partially explain the unnecessary role of endogenous TNF-α, which is known to induce apoptosis (37).

It is well known that the production of IFN-γ by the adaptive T cell response is necessary for immune protection against TB (65–67). In addition, it has been shown that IFN-γ is produced by cells of the innate immune system, including NK cells (18) and murine macrophages (20, 21), in response to IL-12+IL-18, as well as in response to mycobacterial infection in human macrophages (19). A surprising finding of the current study was that the ability of IL-12+IL-18 to induce, in macrophages, the autonomous production of IFN-γ, a cytokine typically associated with the adaptive T cell response, was required for generation of an innate antimicrobial response. We found that treatment of macrophages with IL-12 and IL-18 induced IL-15 and IL-32, which prompted the activation of the VDR leading to upregulation of antimicrobial peptides known to inhibit the growth of intracellular mycobacteria, the identical pathway induced by exogenous treatment with IFN-γ (32). Evidence for this vital role of IFN-γ was demonstrated by experiments in which neutralizing IFN-γ blockade abrogated the IL-12+IL-18–induced production of IL-15 and IL-32, as well as the antimycobacterial response. These data provide new insight into the mechanism of innate antimicrobial activity against *M. tuberculosis* in human macrophages, delineated as IL-12+IL-18 → IFN-γ → IL-15/IL-32 → antimicrobial peptides and autophagy. Furthermore, innate and adaptive immune responses have evolved to have distinct functions, yet both have retained the ability to produce IFN-γ to activate the vitamin D–dependent antimicrobial pathway (32). It is noteworthy that IL-12+IL-18 activation of IFN-γ was not inhibited by IFN-β. This finding suggests that the combined action of IL-12+IL-18 can override the immunosuppressive action of type I IFNs, which are upregulated as part of the pathogenesis of TB and have been shown to negatively regulate the host immune defense against bacterial infection (37, 47, 68). One mechanism by which type I IFNs downregulate IFN-γ–induced immune responses is via the production of IL-10 (47).

IL-12+IL-18–induced restriction of mycobacteria in pulmonary epithelial cells does not involve the production of IFN-γ or IL-15, as seen in the setting of mycobacteria-infected macrophages. However, the restriction of intracellular mycobacterial growth in lung epithelial cells requires VDR-derived antimicrobial peptides and autophagy, suggesting the existence of an alternate pathway for the induction of these host defense responses. Importantly, IL-12+IL-18–mediated activation of p38/STAT4 can activate VDR, VDR-derived CAMP, and autophagy, leading to inhibition of intracellular mycobacterial growth in macrophage and pulmonary epithelial cell lineages.

IL-12+IL-18 cosignaling does not seem to upregulate TNF-α production, and neutralizing anti–TNF-α Ab blockade fails to reduce or abrogate the IL-12+IL-18–mediated growth inhibition of intracellular mycobacteria. Several points might help to explain the lack of a role for TNF-α in the IL-12+IL-18 restriction of intracellular mycobacteria. First, an absence of changes in low TNF-α baseline after IL-12+IL-18 treatment suggests that low-level TNF-α does not contribute to the intrinsic function of the IL-12+IL-18–induced growth inhibition of mycobacteria. We found that concentrations of exogenous TNF-α ≥ 50 ng/ml mediated the inhibition of intracellular mycobacteria (data not shown). Second, in vitro blockade of the TNF-α signal might not be sufficient to uncover upregulation of intracellular mycobacteria growth. In fact, intracellular mycobacterial growth could not be enhanced by three well-documented TNF-α–blockade drugs capable of reactivating latent TB (69). Third, in vitro TNF-α–mediated growth inhibition of intracellular mycobacteria appears to require granzyme A or other factors produced by T cells, and such cooperative TNF-α inhibition is independent of autophagy

(41). In contrast, IL-12+IL-18–induced inhibition involves VDR induction of cathelicidin, β-defensin 2, and autophagy, without the need for granzyme A.

Whether the IL-12+IL-18–induced antimicrobial pathway is active in vivo appears to be complex and beyond the scope of the current study. Natural *M. tuberculosis* infection of humans certainly induces the production of IL-12/IL-18 (11, 15), and it will be interesting to determine whether IL-12/IL-18 rapidly produced upon *M. tuberculosis* exposure can help to control infection or even sterilizing immunity. In this natural setting, a much lower concentration of IL-12+IL-18 may be sufficient for *M. tuberculosis* growth restriction.

Thus, treatment of macrophages and lung epithelial cells with IL-12+IL-18 induced an antimicrobial response, providing evidence for a mechanism involving the p38–MAPK and STAT4 signaling pathways, the production of the antimicrobial peptide cathelicidin, as well as enhanced induction of autophagy. Furthermore, IL-12+IL-18 treatment led to the production of IFN-γ and the induction of IL-32 and *IL-15* in human macrophages. Defining this novel mechanism may also provide innovative treatment strategies for blocking further transmission of *M. tuberculosis* and reactivation of TB in the already-infected population.

Acknowledgments

We thank members of the Chen Laboratory for technical support.

Disclosures

The authors have no financial conflicts of interest.

References

1. Wickremasinghe, M. I., L. H. Thomas, and J. S. Friedland. 1999. Pulmonary epithelial cells are a source of IL-8 in the response to *Mycobacterium tuberculosis*: essential role of IL-1 from infected monocytes in a NF-kappa B-dependent network. *J. Immunol.* 163: 3936–3947.
2. Diamond, G., D. Legarda, and L. K. Ryan. 2000. The innate immune response of the respiratory epithelium. *Immunol. Rev.* 173: 27–38.
3. Roy, S., S. Sharma, M. Sharma, R. Aggarwal, and M. Bose. 2004. Induction of nitric oxide release from the human alveolar epithelial cell line A549: an in vitro correlate of innate immune response to *Mycobacterium tuberculosis*. *Immunology* 112: 471–480.
4. Chuquimia, O. D., D. H. Petursdottir, M. J. Rahman, K. Hartl, M. Singh, and C. Fernández. 2012. The role of alveolar epithelial cells in initiating and shaping pulmonary immune responses: communication between innate and adaptive immune systems. *PLoS One* 7: e32125.
5. Chuquimia, O. D., D. H. Petursdottir, N. Periolo, and C. Fernández. 2013. Alveolar epithelial cells are critical in protection of the respiratory tract by secretion of factors able to modulate the activity of pulmonary macrophages and directly control bacterial growth. *Infect. Immun.* 81: 381–389.
6. World Health Organization. Global Tuberculosis Report 2017. Available at: http://www.who.int/tb/publications/global_report/en/. Accessed: January 19, 2018.
7. Cooper, A. M. 2009. Cell-mediated immune responses in tuberculosis. *Annu. Rev. Immunol.* 27: 393–422.
8. Stenger, S., and R. L. Modlin. 1999. T cell mediated immunity to *Mycobacterium tuberculosis*. *Curr. Opin. Microbiol.* 2: 89–93.
9. Megyeri, K., K. Buzás, A. Miczák, E. Buzás, L. Kovács, G. Seprényi, A. Falus, and Y. Mándi. 2006. The role of histamine in the intracellular survival of *Mycobacterium bovis* BCG. *Microbes Infect.* 8: 1035–1044.
10. Henderson, R. A., S. C. Watkins, and J. L. Flynn. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol.* 159: 635–643.
11. Zhang, M., M. K. Gately, E. Wang, J. Gong, S. F. Wolf, S. Lu, R. L. Modlin, and P. F. Barnes. 1994. Interleukin 12 at the site of disease in tuberculosis. *J. Clin. Invest.* 93: 1733–1739.
12. Sieling, P. A., X. H. Wang, M. K. Gately, J. L. Oliveros, T. McHugh, P. F. Barnes, S. F. Wolf, L. Golkar, M. Yamamura, Y. Yogi, et al. 1994. IL-12 regulates T helper type 1 cytokine responses in human infectious disease. *J. Immunol.* 153: 3639–3647.
13. Munk, M. E., P. Mayer, P. Anding, K. Feldmann, and S. H. Kaufmann. 1996. Increased numbers of interleukin-12-producing cells in human tuberculosis. *Infect. Immun.* 64: 1078–1080.
14. Fulton, S. A., J. M. Johnsen, S. F. Wolf, D. S. Sieburth, and W. H. Boom. 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. *Infect. Immun.* 64: 2523–2531.
15. Vankayalapati, R., B. Wizel, S. E. Weis, B. Samten, W. M. Girard, and P. F. Barnes. 2000. Production of interleukin-18 in human tuberculosis. *J. Infect. Dis.* 182: 234–239.
16. Giacomini, E., E. Iona, L. Ferroni, M. Miettinen, L. Fattorini, G. Orefici, I. Julkunen, and E. M. Coccia. 2001. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J. Immunol.* 166: 7033–7041.
17. Tominaga, K., T. Yoshimoto, K. Torigoe, M. Kurimoto, K. Matsui, T. Hada, H. Okamura, and K. Nakanishi. 2000. IL-12 synergizes with IL-18 or IL-1beta for IFN-gamma production from human T cells. *Int. Immunol.* 12: 151–160.
18. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechser, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.
19. Fenton, M. J., M. W. Vermeulen, S. Kim, M. Burdick, R. M. Strieter, and H. Kornfeld. 1997. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. *Infect. Immun.* 65: 5149–5156.
20. Munder, M., M. Mallo, K. Eichmann, and M. Modolell. 1998. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. *J. Exp. Med.* 187: 2103–2108.
21. Schindler, H., M. B. Lutz, M. Röllinghoff, and C. Bogdan. 2001. The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J. Immunol.* 166: 3075–3082.
22. Shen, H., F. Wang, G. Zeng, L. Shen, H. Cheng, D. Huang, R. Wang, L. Rong, and Z. W. Chen. 2016. Bis-biguanide dihydrochloride inhibits intracellular replication of *M. tuberculosis* and controls infection in mice. *Sci. Rep.* 6: 32725.
23. Xu, Y., C. Jagannath, X. D. Liu, A. Sharafkhaneh, K. E. Kolodziejska, and N. T. Eissa. 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* 27: 135–144.
24. Zeng, G., C. Y. Chen, D. Huang, S. Yao, R. C. Wang, and Z. W. Chen. 2011. Membrane-bound IL-22 after de novo production in tuberculosis and anti-*Mycobacterium tuberculosis* effector function of IL-22+ CD4+ T cells. *J. Immunol.* 187: 190–199.
25. Qiu, Y., J. Chen, H. Liao, Y. Zhang, H. Wang, S. Li, Y. Luo, D. Fang, G. Li, B. Zhou, et al. 2012. Tim-3-expressing CD4+ and CD8+ T cells in human tuberculosis (TB) exhibit polarized effector memory phenotypes and stronger anti-TB effector functions. *PLoS Pathog.* 8: e1002984.
26. Sharma, M., S. Sharma, S. Roy, S. Varma, and M. Bose. 2007. Pulmonary epithelial cells are a source of interferon-gamma in response to *Mycobacterium tuberculosis* infection. *Immunol. Cell Biol.* 85: 229–237.
27. Stokes, R. W., and D. Doxsee. 1999. The receptor-mediated uptake, survival, replication, and drug sensitivity of *Mycobacterium tuberculosis* within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. *Cell. Immunol.* 197: 1–9.
28. Méndez-Samperio, P., E. Miranda, and A. Trejo. 2008. Expression and secretion of cathelicidin LL-37 in human epithelial cells after infection by *Mycobacterium bovis* bacillus Calmette-Guérin. *Clin. Vaccine Immunol.* 15: 1450–1455.
29. Méndez-Samperio, P., E. Miranda, and A. Trejo. 2008. Regulation of human beta-defensin-2 by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG): involvement of PKC, JNK, and PI3K in human lung epithelial cell line (A549). *Peptides* 29: 1657–1663.
30. Shin, D. M., and E. K. Jo. 2011. Antimicrobial peptides in innate immunity against mycobacteria. *Immune Netw.* 11: 245–252.
31. Liu, P. T., M. Schenk, V. P. Walker, P. W. Dempsey, M. Kanchanapoomi, M. Wheelwright, A. Vazirnia, X. Zhang, A. Steinmeyer, U. Zügel, et al. 2009. Convergence of IL-1beta and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS One* 4: e5810.
32. Fabri, M., S. Stenger, D. M. Shin, J. M. Yuk, P. T. Liu, S. Realegeno, H. M. Lee, S. R. Krutzik, M. Schenk, P. A. Sieling, et al. 2011. Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages. *Sci. Transl. Med.* 3: 104ra102.
33. Bloom, B. R., and R. L. Modlin. 2016. Mechanisms of defense against intracellular pathogens mediated by human macrophages. *Microbiol. Spectr.* DOI: 10.1128/microbiolspec.MCHD-0006-2015.
34. Lee, J. K., S. H. Kim, E. C. Lewis, T. Azam, L. L. Reznikov, and C. A. Dinarello. 2004. Differences in signaling pathways by IL-1beta and IL-18. *Proc. Natl. Acad. Sci. USA* 101: 8815–8820.
35. Chandrasekar, B., S. Mummidi, A. J. Valente, D. N. Patel, S. R. Bailey, G. L. Freeman, M. Hatano, T. Tokuhisa, and L. E. Jensen. 2005. The pro-atherogenic cytokine interleukin-18 induces CXCL16 expression in rat aortic smooth muscle cells via MyD88, interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor 6, c-Src, phosphatidylinositol 3-kinase, Akt, c-Jun N-terminal kinase, and activator protein-1 signaling. *J. Biol. Chem.* 280: 26263–26277.
36. Bacon, C. M., E. F. Petricoin, III, J. R. Ortaldo, R. C. Rees, A. C. Larner, J. A. Johnston, and J. J. O'Shea. 1995. Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. *Proc. Natl. Acad. Sci. USA* 92: 7307–7311.
37. Xu, G., J. Wang, G. F. Gao, and C. H. Liu. 2014. Insights into battles between *Mycobacterium tuberculosis* and macrophages. *Protein Cell* 5: 728–736.
38. Guo, X. G., T. X. Ji, Y. Xia, and Y. Y. Ma. 2013. Autophagy protects type II alveolar epithelial cells from *Mycobacterium tuberculosis* infection. *Biochem. Biophys. Res. Commun.* 432: 308–313.
39. Fine, K. L., M. G. Metcalfe, E. White, M. Virji, R. K. Karls, and F. D. Quinn. 2012. Involvement of the autophagy pathway in trafficking of *Mycobacterium*

tuberculosis bacilli through cultured human type II epithelial cells. *Cell. Microbiol.* 14: 1402–1414.
40. Blommaart, E. F., U. Krause, J. P. Schellens, H. Vreeling-Sindelárová, and A. J. Meijer. 1997. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* 243: 240–246.
41. Spencer, C. T., G. Abate, I. G. Sakala, M. Xia, S. M. Truscott, C. S. Eickhoff, R. Linn, A. Blazevic, S. S. Metkar, G. Peng, et al. 2013. Granzyme A produced by γ(9)δ(2) T cells induces human macrophages to inhibit growth of an intracellular pathogen. *PLoS Pathog.* 9: e1003119.
42. Zhu, H., H. O. Fearnhead, and G. M. Cohen. 1995. An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *FEBS Lett.* 374: 303–308.
43. Bai, X., C. A. Dinarello, and E. D. Chan. 2015. The role of interleukin-32 against tuberculosis. *Cytokine* 76: 585–587.
44. Douvas, G. S., D. L. Looker, A. E. Vatter, and A. J. Crowle. 1985. Gamma interferon activates human macrophages to become tumoricidal and leishmanicidal but enhances replication of macrophage-associated mycobacteria. *Infect. Immun.* 50: 1–8.
45. Kumar, D., L. Nath, M. A. Kamal, A. Varshney, A. Jain, S. Singh, and K. V. Rao. 2010. Genome-wide analysis of the host intracellular network that regulates survival of *Mycobacterium tuberculosis. Cell* 140: 731–743.
46. Bouchonnet, F., N. Boechat, M. Bonay, and A. J. Hance. 2002. Alpha/beta interferon impairs the ability of human macrophages to control growth of *Mycobacterium* bovis BCG. *Infect. Immun.* 70: 3020–3025.
47. Teles, R. M., T. G. Graeber, S. R. Krutzik, D. Montoya, M. Schenk, D. J. Lee, E. Komisopoulou, K. Kelly-Scumpia, R. Chun, S. S. Iyer, et al. 2013. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. *Science* 339: 1448–1453.
48. García, V. E., K. Uyemura, P. A. Sieling, M. T. Ochoa, C. T. Morita, H. Okamura, M. Kurimoto, T. H. Rea, and R. L. Modlin. 1999. IL-18 promotes type 1 cytokine production from NK cells and T cells in human intracellular infection. *J. Immunol.* 162: 6114–6121.
49. Sugawara, I., H. Yamada, H. Kaneko, S. Mizuno, K. Takeda, and S. Akira. 1999. Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. *Infect. Immun.* 67: 2585–2589.
50. Yoshimoto, T., K. Takeda, T. Tanaka, K. Ohkusu, S. Kashiwamura, H. Okamura, S. Akira, and K. Nakanishi. 1998. IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN-gamma production. *J. Immunol.* 161: 3400–3407.
51. Fantuzzi, L., P. Puddu, B. Varano, M. Del Cornò, F. Belardelli, and S. Gessani. 2000. IFN-alpha and IL-18 exert opposite regulatory effects on the IL-12 receptor expression and IL-12-induced IFN-gamma production in mouse macrophages: novel pathways in the regulation of the inflammatory response of macrophages. *J. Leukoc. Biol.* 68: 707–714.
52. Thada, S., M. Ponnana, R. Sivangala, L. Joshi, M. Alasandagutti, M. S. Ansari, R. R. Schumann, V. Valluri, and S. Gaddam. 2016. Polymorphisms of IFN-γ (+874A/T) and IL-12 (+1188A/C) in tuberculosis patients and their household contacts in Hyderabad, India. *Hum. Immunol.* 77: 559–565.
53. Han, M., J. Yue, Y. Y. Lian, Y. L. Zhao, H. X. Wang, and L. R. Liu. 2011. Relationship between single nucleotide polymorphism of interleukin-18 and susceptibility to pulmonary tuberculosis in the Chinese Han population. *Microbiol. Immunol.* 55: 388–393.
54. Montoya, D., M. S. Inkeles, P. T. Liu, S. Realegeno, R. M. Teles, P. Vaidya, M. A. Munoz, M. Schenk, W. R. Swindell, R. Chun, et al. 2014. IL-32 is a molecular marker of a host defense network in human tuberculosis. *Sci. Transl. Med.* 6: 250ra114.
55. Krutzik, S. R., M. Hewison, P. T. Liu, J. A. Robles, S. Stenger, J. S. Adams, and R. L. Modlin. 2008. IL-15 links TLR2/1-induced macrophage differentiation to the vitamin D-dependent antimicrobial pathway. *J. Immunol.* 181: 7115–7120.
56. Shin, D. M., J. M. Yuk, H. M. Lee, S. H. Lee, J. W. Son, C. V. Harding, J. M. Kim, R. L. Modlin, and E. K. Jo. 2010. Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling. *Cell. Microbiol.* 12: 1648–1665.
57. Alonso, S., K. Pethe, D. G. Russell, and G. E. Purdy. 2007. Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy. *Proc. Natl. Acad. Sci. USA* 104: 6031–6036.
58. Watson, R. O., P. S. Manzanillo, and J. S. Cox. 2012. Extracellular *M. tuberculosis* DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell* 150: 803–815.
59. Gutierrez, M. G., S. S. Master, S. B. Singh, G. A. Taylor, M. I. Colombo, and V. Deretic. 2004. Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119: 753–766.
60. Klug-Micu, G. M., S. Stenger, A. Sommer, P. T. Liu, S. R. Krutzik, R. L. Modlin, and M. Fabri. 2013. CD40 ligand and interferon-γ induce an antimicrobial response against *Mycobacterium tuberculosis* in human monocytes. *Immunology* 139: 121–128.
61. Jayaraman, P., I. Sada-Ovalle, T. Nishimura, A. C. Anderson, V. K. Kuchroo, H. G. Remold, and S. M. Behar. 2013. IL-1β promotes antimicrobial immunity in macrophages by regulating TNFR signaling and caspase-3 activation. *J. Immunol.* 190: 4196–4204.
62. Chen, M., M. Divangahi, H. Gan, D. S. Shin, S. Hong, D. M. Lee, C. N. Serhan, S. M. Behar, and H. G. Remold. 2008. Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. *J. Exp. Med.* 205: 2791–2801.
63. Davis, J. M., and L. Ramakrishnan. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136: 37–49.
64. Blomgran, R., L. Desvignes, V. Briken, and J. D. Ernst. 2012. *Mycobacterium tuberculosis* inhibits neutrophil apoptosis, leading to delayed activation of naive CD4 T cells. *Cell Host Microbe* 11: 81–90.
65. Bustamante, J., S. Boisson-Dupuis, L. Abel, and J. L. Casanova. 2014. Mendelian susceptibility to mycobacterial disease: genetic, immunological, and clinical features of inborn errors of IFN-γ immunity. *Semin. Immunol.* 26: 454–470.
66. Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. A. Stewart, and B. R. Bloom. 1993. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178: 2249–2254.
67. Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J. Exp. Med.* 178: 2243–2247.
68. Du, Q., J. Xie, H. J. Kim, and X. Ma. 2013. Type I interferon: the mediator of bacterial infection-induced necroptosis. *Cell. Mol. Immunol.* 10: 4–6.
69. Saliu, O. Y., C. Sofer, D. S. Stein, S. K. Schwander, and R. S. Wallis. 2006. Tumor-necrosis-factor blockers: differential effects on mycobacterial immunity. *J. Infect. Dis.* 194: 486–492.