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IL-1β induces the rapid secretion of the antimicrobial protein IL-26 from Th17 cells

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Abstract

Th17 cells play a critical role in the adaptive immune response against extracellular bacteria and the possible mechanisms by which they can protect against infection are of particular interest. Here we describe a novel IL-1β dependent pathway for secretion of the antimicrobial peptide IL-26 from human Th17 cells that is independent of and more rapid than classical T cell receptor activation. We find that IL-26 is secreted by 3 hours after treating peripheral blood mononuclear cells with *Mycobacterium leprae*, as compared to 48 hours for IFN-γ and IL-17A. IL-1β was required for microbial ligand induction of IL-26 and was sufficient to stimulate IL-26 release from Th17 cells. Only the IL-1 receptor 1 (IL-1RI) positive Th17 cells responded to IL-1β, inducing an NF-kB regulated transcriptome. Finally, supernatants from IL-1β treated memory T cells killed *E. coli* in an IL-26 dependent manner. These results identify a mechanism by which human IL-1RI^{+} "antimicrobial Th17 cells" can be rapidly activated by IL-1β as part of the innate immune response to produce IL-26 in order to kill extracellular bacteria.

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Data and materials availability

The RNA-seq data has been uploaded to the Gene Expression Omnibus under accession number GSE127457 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE127457).

Disclosures

We have no financial interests to disclose.

Introduction

T cells are crucial for effective host defense against a wide range of infectious pathogens, triggering an antimicrobial activity against the foreign invader. T cells release antimicrobial proteins, which directly kill the invading pathogen such as granulysin (1), or cytokines that act indirectly, by causing the induction of antimicrobial effector molecules in other cell types. It is generally accepted that Th17 cells play a critical role in host defense against extracellular bacteria (2–5). The IL-17 family of cytokines, including IL-17A and IL-17F, bind to receptors on a variety of cell types including epithelial cells, fibroblasts, macrophages, and neutrophils. This leads to the secretion of chemokines like CXCL2, CXCL5, and IL-8 and inflammatory cytokines IL-6, G-CSF and GM-CSF, attracting and activating myeloid cells at the site of infection (6–8). IL-17 and IL-22 from Th17 cells are also able to stimulate antimicrobial defensin secretion from epithelial cells (4, 7–9). We have previously reported that Th17 cells also secrete IL-26, a cytokine that has been shown to be directly antimicrobial against a variety of Gram positive and Gram negative bacteria (9).

IL-26, a 19-kDa α -helical protein that belongs to the IL-10 cytokine family, is conserved in most vertebrate species, but is absent in mice (10). It is secreted primarily by activated T cells, and among $CD4^+$ helper T cells it is more specifically produced by Th17 cells (9, 11, 12). IL-26 displays certain hallmarks of naturally occurring antimicrobial peptides, including amphipathic structure, clustering of cationic charges, and multimer formation, and was found to disrupt bacterial membranes via pore formation (13). Aside from its antimicrobial properties, IL-26 is also pro-inflammatory, and signaling through its IL-10R2/IL-20R1 heterodimeric receptor on epithelial cells results in secretion of proinflammatory cytokines such as IL-6, IL-1β, and IL-8 (14, 15). Monocytes and macrophages may also respond to IL-26 to produce inflammatory cytokines despite only expressing the IL-10R2 subunit of the receptor (16). Furthermore, IL-26 has been shown to bind to both DNA and RNA and enhance their detection by cellular sensors, resulting in increased type I interferon production (9, 17).

Compared to the rapidity of the innate response, the induction of adaptive T cell effector function is relatively slow, preventing T cells from contributing to host defense during the critical early phase of the immune response (18). There is evidence that some lymphoid cell subpopulations can be rapidly activated by cytokines to contribute to the early immune response, such as innate lymphoid cells (ILCs), which are devoid of T cell receptors (TCR), and γδ T cells (19–21). In addition, some of the T cell polarizing cytokines are known to augment activation of differentiated cells. For example, IL-12, which polarize naïve cells to become Th1 cells (22–24), synergizes with IL-18 to induce IFN-γ production by Th1 cells in the absence of TCR activation (25) and IL-1β, which is a polarizing cytokine for Th17 cells (12, 26, 27), enhances TCR induced release of IL-17 from Th17 cells (28). Here we investigated whether Th17 cells can be activated by TCR-independent pathways to secrete the antimicrobial protein IL-26 to defend the host against microbial infection.

Materials and Methods

Study design

This study was aimed at identifying alternative pathways of IL-26 release from Th17 cells that may contribute to host defense against bacterial infection. Human peripheral blood was obtained from healthy donors at the University of California, Los Angeles (UCLA) and from leprosy donors at the University of Southern California. Researchers were not blinded as to the source of the blood samples. Written informed consent was provided by all donors according to institutional review board protocols of both institutions.

Leprosy PBMC stimulation with *M. leprae* sonicate

Whole blood specimens from leprosy patients were obtained through collaboration with the Hansen's Disease Clinic at Los Angeles County/University of Southern California Medical Center. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood via density centrifugation with Ficoll-Paque solution (GE Healthcare Life Sciences). PBMCs were cultured in RPMI 1640 with 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2mM), and sodium pyruvate (1 mM). 1×10^6 PBMCs were plated per well in 24 well plates at a density of 1×10^6 cells/ml and stimulated or not with 10 μg/ml sonicated *M. leprae*. Cell free supernatants were collected after various timepoints for analysis with ELISA kits for IL-26 (Cusabio Biotech), IL-17A, and IFN-γ (DuoSet, R&D Systems)

Stimulation of healthy donor PBMCs with PRR ligands and cytokines

PBMCs were isolated from whole blood of healthy donors who provided written informed consent and were cultured as described above. The cells were stimulated or not with the following PRR ligands (Invivogen): 100 ng/ml Pam3CSK4 (TLR2/1), 1×10^7 cells/ml heat killed *Listeria monocytogenes* (TLR2), 1 μg/ml high molecular weight poly(I:C) (TLR3), 1 μg/ml low molecular weight poly(I:C) (TLR3), 100 ng/ml lipopolysaccharide (TLR4), 100 ng/ml flagellin (TLR5), 100 ng/ml FSL-1 (TLR2/6), 1 μg/ml imiquimod (TLR7/8), 100 ng/ml ssRNA/LyoVec (TLR8), 1.25 μM dsDNA (TLR9), and 10 ng/ml muramyl dipeptide (NOD2). Cell free supernatants were collected after 16 hours and assayed for cytokine secretion by ELISA. In some experiments, PBMCs were stimulated with a suboptimal dose of 1 ng/ml Pam3CSK4 (TLR2/1L) in the presence or absence of 40 μg/ml of anti-IL-1β blocking antibodies or 40 μg/ml isotype matched control antibody (Invivogen). For cytokine treatment of healthy donor PBMCs, cells were cultured as above with or without 20 ng/ml of recombinant IL-1β, IL-6, IL-23, TGF-β, IL-12, IL-18, IL-4, IL-15, or TNF-α (BioLegend). Cell free supernatants were collected after 48 hours.

Isolation and stimulation of T cell subsets

T cell subsets were isolated from PBMC with the human T cell isolation kit, human $CD4^+$ T cell isolation kit, human naïve $CD4^+$ T cell isolation kit, and human memory $CD4^+$ T cell enrichment kits (EasySep, StemCell Inc.) and cultured as above. The isolated T cell subsets were routinely of ≥ 97% purity. Isolated $CD3^+$ T cells, $CD4^+$ T cells, naïve $CD4^+$ T cells, and memory $CD4^+$ T cells were stimulated or not with IL-1β, IL-6, or IL-23. Cytokine

concentrations and stimulation times varied per experiment, as indicated in the figure legends. For TCR stimulation, varying amounts of Immunocult human T cell activator (StemCell Inc.) were added to the cell culture as indicated in the text and figure legends. Cell free supernatants were collected before analysis of cytokine secretion with ELISA kits for IL-26 (Cusabio), IL-22, IL-17A, IL-17F, and IL-8 (DuoSet, R&D systems).

Sorting memory CD4$^+$ T cell subsets.

Immunomagnetically isolated memory CD4$^+$ T cells were stained with antibodies against APC- or BV421-CCR6 (BioLegend, clone 29–2L17), PE-CD161 (BioLegend, clone HP-3G10), FITC-CXCR3 (BioLegend, clone G025H7), and PECy7-CCR4 (BioLegend, clone L291H4) and sorted using FACS into Th17 cells (CCR6$^+$CD161$^+$CCR4$^+$CXCR3$^-$), Th1 cells (CCR6$^-$CD161$^-$CCR4$^-$CXCR3$^+$), and Th2 cells (CCR6$^-$CD161$^-$CCR4$^+$CXCR3$^-$). The gating strategy was to first gate on CCR6$^+$CD161$^+$ (for Th17) or CCR6$^-$CD161$^-$ (for Th1 and Th2), and subsequently gate on CCR4$^+$CXCR3$^-$ (for Th17 and Th2) or CCR4$^-$CXCR3$^+$ (for Th1). In some experiments, memory CD4$^+$ T cells were incubated overnight with 1 nM recombinant human IL-2 and stained additionally with antibodies against APC-IL-1RI (R&D Systems, polyclonal goat anti-human) to sort IL-1RI$^+$ Th17 cells (CCR6$^+$CD161$^+$CCR4$^+$CXCR3$^-$IL-1RI$^+$) and IL-1RI$^-$ Th17 cells (CCR6$^+$CD161$^+$CCR4$^+$CXCR3$^-$IL-1RI$^-$).

Stimulation of sorted memory CD4$^+$ T cell subsets.

Th1, Th2, and Th17 cells were plated in 24 well plates at a density of 3×10^5 cells/ml in one ml of RPMI 1640 with 10% fetal calf serum, penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2mM), and sodium pyruvate (1 mM).). Because of the yield from flow sorting, we plated a lower density of the Th subsets as compared to memory CD4+ T cells. The cells were stimulated with 20 ng/ml IL-1β for 48 hours and cell free supernatants were collected for ELISA. IL-1RI$^+$ and IL-1RI$^-$ Th17 cells were sorted and plated in 200 ul at a density 1×10^6 cells/ml in round bottom 96 well plates. In this experiment, we recovered the Th17 subpopulations from an eluted whole blood leukoreduction filter (Trima Accel) in order to increase cell yield. The cells were stimulated or not with 100 ng/ml IL-1β or 5 μl/ml of Immunocult human T cell activator for 9 hours, as cells were also harvested for RNA sequencing. Cell free supernatants were collected for ELISA analysis.

RNA sequencing of IL-1RI$^+$ and IL-1RI$^-$ Th17 cells

IL-1RI$^+$ and IL-1RI$^-$ Th17 cells were stimulated for 9 hours as described above before cell pellets were lysed with RLT buffer and RNA extracted as per Qiagen RNEasy micro kit instructions (Qiagen). Sequencing libraries were prepared by the Technology Center for Genomics & Bioinformatics at UCLA using Illumina TruSeq Stranded Total RNA Sample Prep kit and sequenced by single end sequencing on an Illumina HiSeq3000.

Analysis of RNA-seq data

The reads were mapped with STAR 2.5.3a to the human genome (Hg38). The counts for each gene were obtained by using --quantMode GeneCounts in STAR(29) commands, and the other parameters during alignment were set to default. Differential expression analyses

were carried out using DESeq2 (30). Normalized counts were obtained using DESeq2 rlog function with default parameters. Principle component analysis and hierarchical clustering were performed on the normalized counts in R. Functional analysis was performed using Ingenuity Pathway Analysis software (Qiagen). Genes that were significantly upregulated by IL-1β vs media in IL-1RI$^+$ Th17 cells with a FC ≥ 1.5 and p-adj ≤ 0.05 were uploaded and core expression analysis was performed. Upstream transcription factor analysis was performed using the Enrichr website (http://amp.pharm.mssm.edu/Enrichr/).

TCR gene analysis

MiXCR software was used for direct extraction of TCR CDR3 sequences from RNA-Seq datasets. Analyses were performed with "-p rna-seq" option, as recommended for analysis of RNA-seq data (31). Scripts developed in R (32) were then used to parse the alignment files and perform aggregate counts of the number of reads per TCR gene segment and TCR gene segment combination (33).

NF-kB signaling pathway inhibition

Immunomagnetically isolated memory CD4$^+$ T cells were cultured as described above and stimulated with 20 ng/ml IL-1β or 0.5 μl/ml Immunocult for 48 hours in the presence or absence of 10 μM Bay 11–7082 (Millipore-Sigma). Cell free supernatants were collected and IL-26 concentrations were measured by ELISA.

Antimicrobial assays

Immunomagnetically purified memory CD4$^+$ T cells were cultured in RPMI 1640 with 10% fetal calf serum, L-glutamine (2mM), and sodium pyruvate (1 mM), with or without 20 ng/ml IL-1β for 48 hours and cell free supernatants were collected. *Escherichia coli* was streaked on LB agar and grown at 37°C overnight. A single colony was picked and inoculated into LB broth, which was incubated overnight at 37°C and 250 rpm shaking. Cell number was determined via OD600. 1×10^2 bacteria were inoculated into 100 ul of T cell supernatant, with or without 20 μg/ml blocking anti-IL-26 antibodies or isotype control antibodies (R&D systems), or into unconditioned media containing either 20 ng/ml recombinant IL-1β protein, a combination of 50 u/ml penicillin and 50 μg/ml streptomycin, or nothing and incubated overnight at 37°C and 250 rpm shaking. Increasing dilutions of the cultures were plated on LB agar plates and after growth overnight at 37°C viability was assessed by counting colony forming units.

Statistical analysis

Statistical analyses were calculated using GraphPad Prism version 8.0, and p-values ≤ 0.05 were assigned as significant. Each figure legend contains the specific statistical test performed, which were chosen based on the number of comparison groups and normality of the data. For comparisons involving two groups, Paired Student's t-test or one-way ANOVA with Sidak's post-test was performed. For more than two comparison groups, one-way ANOVA with Tukey's post-test, two-way ANOVA with Tukey's post-test, or Friedman's test with Dunn's post-test were performed. Data are represented in figures as mean ± SEM.

Results

IL-26 secretion is rapid after PBMC stimulation with bacterial sonicate

In order to determine the kinetics of IL-26 production in response to invading bacteria, we studied the time course of cytokine release from PBMCs in response to *Mycobacterium leprae*, given that this bacterium, in addition to inducing Th1 cells, activates Th17 cells (34, 35). We measured the kinetics of IL-26 induction compared with characteristic memory T cell cytokines IL-17A and IFN-γ by stimulating PBMCs from tuberculoid leprosy (T-lep) patients with sonicated *M. leprae*, as these individuals mount strong T cell responses to the pathogen (36). IL-17A and IFN-γ were not detectable until 2 days after *M. leprae* treatment, as is typical for a memory response (Fig. 1A). Surprisingly, IL-26 was detected in the supernatants as early as 3 hours with concentrations steadily increasing over 4 days. These results indicate that *M. leprae* sonicate rapidly induces IL-26 at an earlier time point than a classic memory recall response.

Innate stimuli trigger IL-26 secretion from PBMC in an IL-1β dependent manner

We next investigated the innate mechanism of IL-26 induction by stimulating PBMCs overnight with a variety of microbial ligands that activate pattern recognition receptors (PRRs), given that mycobacteria express a range of innate ligands. The early secretion of IL-26 by PBMCs in response to *M. leprae* sonicate suggested a mechanism involving an alternative pathway not requiring T cell memory, such that we studied IL-26 induction in healthy donors. We found several PRR ligands that significantly induced IL-26 in high (TLR5L, TLR4L, TLR2/1L, TLR2L), medium (TLR2/6L, NOD2L) and low (TLR8L, TLR9L) amounts (Fig. 1B).

We asked whether PRR induction of IL-26 was a direct effect of PRR engagement or was dependent upon the release of a proinflammatory cytokine. To this end we investigated whether known PRR induced cytokines, as well as T cell modulatory cytokines, were sufficient by themselves to induce IL-26 (Fig. 1C). Strikingly, of nine cytokines tested, only IL-1β was able to induce significant IL-26 secretion from the T cells after 48 hours. Furthermore, analysis of supernatants collected from PRR ligand stimulated PBMC revealed that PRR ligands that stimulated greater amounts of IL-1β also tended to stimulate greater amounts of IL-26 (Fig. 1D). We were able to fit a sigmoidal dose-response curve to this data with an $R^2 = 0.69$, which demonstrated a clear association between IL-1β and IL-26 secretion. Finally, using a suboptimal dose of Pam3CSK4, a TLR2/1 ligand, we find that the addition of anti-IL-1β blocking antibody led to a significant decrease in IL-26 secretion after PBMC stimulation (Fig. 1E). These results demonstrate that the mechanism for the early induction of IL-26 by PRR ligands is mediated by the production of IL-1β.

Memory $CD4^+$ T cells secrete IL-26 in response to IL-1β in the absence of T cell receptor activation

Given that PBMCs are a heterogeneous group of immune cells and that IL-26 secretion is primarily associated with Th17 cells, we asked whether T cells could be induced by IL-1β to secrete IL-26 in the absence of TCR stimulation. We found that when treated with IL-1β, both purified $CD3^+$ T cells and $CD4^+$ T cells produced IL-26, while there was no IL-26

secretion with IL-6 or IL-23 treatment (Fig. 2A). Further experiments demonstrated that purified CD45RO$^+$ memory CD4$^+$ T cells accounted for all of the IL-26 production seen following IL-1β stimulation (Fig. 2B). Within the CD4+ memory T cell compartment, IL-1R1$^+$ T cells were required for the IL-1β-induced IL-26 secretion as depletion of these cells abrogated the response (fig. S1).

Investigation of the response of memory CD4$^+$ T cells to varying doses of IL-1β revealed that after 48 hours, concentrations of IL-1β as low as 0.032 ng/ml resulted in significant levels of IL-26 secretion from the cells compared to untreated cells (p-adj ≤ 0.001) (Fig. 2C, upper left panel). Higher doses of IL-1β resulted in increasing amounts of IL-26 secretion, with the effect plateauing at concentrations of 0.8 ng/ml and above. We also performed a dose response analysis with Immunocult human T cell activator (StemCell Inc.), a solution of tetrameric antibody complexes against CD3 and CD28 (subsequently referred to as anti-CD3/CD28), to compare IL-26 secretion in response to IL-1β or T cell receptor crosslinking (Fig. 2C, upper left panel). The addition of increasing doses of anti-CD3/CD28 led to increasing amounts of IL-26 secretion such that at the two highest concentrations IL-26 secretion was enhanced compared to IL-1β, but this is not statistically significant.

To characterize the kinetics of the IL-26 response, we longitudinally sampled T cell cultures stimulated in parallel with either IL-1β or anti-CD3/CD28 at concentrations that had yielded similar levels of IL-26 after 48 hours (20 ng/ml and 0.5 μl/ml, respectively). When cultured with IL-1β, significant IL-26 secretion became detectable at 6 hours ($p = 0.0003$ as compared to untreated cells), with IL-26 concentrations in the supernatants rising over the course of the next two days (Fig. 2D). In contrast, anti-CD3/CD28 antibody complexes did not stimulate IL-26 secretion at early time points, with protein release only becoming significantly different from untreated control cells after 24 hours of activation ($p \leq 0.0001$). Furthermore, IL-26 secretion was significantly different between IL-1β and anti-CD3/CD28 stimulation at the 6, 9, 12, and 24 hour time points (Fig. 2D). A dose titration of anti-CD3/CD28 was performed at 4 hours to critically measure the early release of IL-26 in response to TCR activation. At this early timepoint, IL-1β at concentrations as low as 0.032 ng/ml induced significant IL-26 in memory CD4$^+$ T cells ($p = 0.002$). The addition of 25 μl/ml of anti-CD3/CD28, a dose that led to a nearly three-fold increase in IL-26 release on average compared to the highest dose of IL-1β after 48 hours, could only induce one ninth of the amount of IL-26 secreted by IL-1β treated cells at 4 hours [631.4 ± 355.1 pg/ml vs. 70.8 ± 58.0 pg/ml]. These data indicate that suboptimal TCR crosslinking was not responsible for the difference in the kinetics of TCR vs. IL-1β induced IL-26 secretion.

Aside from the difference in the rapid IL-26 secretion from memory CD4$^+$ T cells, we also found a functional difference in cytokine secretion between stimulation with IL-1β and anti-CD3/CD28. While both stimuli induced IL-26 secretion, only anti-CD3/CD28 induced the secretion of other Th17 cytokines, including IL-17A, IL-17F, and IL-22 (Fig. 2C). Therefore, these data demonstrate that IL-1β stimulation of memory CD4$^+$ T cells leads to the rapid release of IL-26 but no other Th17 cell cytokines.

IL-26 is secreted by IL-1RI$^+$ Th17 cells

To determine whether IL-1β directly activated Th17 cells, we isolated the various CD4$^+$ T cell populations from memory CD4$^+$ T cells. Using known surface markers for FACS sorting, we evaluated the effect of IL-1β on Th17 cells (CD161$^+$CCR6$^+$CCR4$^+$CXCR3$^-$), Th1 cells (CD161$^-$CCR6$^-$CCR4$^-$CXCR3$^+$), and Th2 cells (CD161$^-$CCR6$^-$CCR4$^+$CXCR3$^-$) (Fig. 3A) (37–39). We found that IL-1β significantly induced IL-26 secretion from only the Th17 cells (Fig. 3B). By flow cytometry, we determined that the memory Th17 cells were Lin$^+$ and did not express CD127 together with CD117, markers of ILC3 cells (40, 41). There are several reports of enriched IL-1 receptor expression on Th17 cells, which we hypothesized would enable these cells to respond to exogenous IL-1β (28, 39, 42), consistent with the finding that IL-1β induction of IL-26 in CD4 T memory cells was abrogated by depletion of IL-1RI$^+$ T cells. To directly determine the response of IL-1RI$^+$ Th17 cells, we sorted IL-1RI$^+$ and IL-1RI$^-$ Th17 cells using FACS (Fig. 3C).. We pre-incubated memory CD4$^+$ T cells with IL-2 overnight before sorting, as IL-2 has been shown to increase the frequency of IL-1RI$^+$ T cells (43). When stimulated with 100 ng/ml IL-1β or 5 μl/ml anti-CD3/CD28 for 9 hours, IL-1RI$^+$ cells secreted IL-26 whereas IL-1RI$^-$ cells did not (Fig. 3D). This finding reflects a previous report that the potential to upregulate *IL26* mRNA after activation and differentiation under Th17 polarizing conditions was dependent upon IL-1RI expression by the treated naïve cells (28). Both populations secreted IL-17A in response to anti-CD3/CD28, although the IL-1RI$^+$ Th17 cells produced more IL-17A, as previously reported (28) (Fig. 3E). Neither cell population secreted IL-17A in response to IL-1β. These results indicate that within Th17 cells, IL-26 production is restricted to the IL-1RI$^+$ subset whether activated by IL-1β or TCR crosslinking. In contrast, IL-17A secretion required TCR activation.

IL-1RI activation induces a predominant NF-κB dependent gene program in Th17 cells

Having demonstrated that IL-1β stimulation of Th17 cells causes secretion of the antimicrobial protein IL-26, we sought to investigate whether IL-1β induced an antimicrobial gene expression program in these cells by mRNA sequencing. We treated sorted IL-1RI$^+$ and IL-1RI$^-$ Th17 cells with either IL-1β, anti-CD3/CD28, or media for 9 hours before harvesting mRNA. An unsupervised principal component analysis of the samples clearly showed that stimulation of both IL-1RI$^+$ and IL-1RI$^-$ Th17 cells with anti-CD3/CD28 had a dramatic effect on gene expression and these samples clustered together in a distinct group away from samples treated with IL-1β or media (Fig. 4A). The IL-1β stimulated and media control samples clustered together, suggesting that the gene expression changes induced by IL-1β were less pronounced compared to anti-CD3/CD28. In each cluster, the IL-1RI$^+$ and IL-1RI$^-$ Th17 cells were mixed.

A second unsupervised analysis, hierarchical clustering, revealed a similar pattern to the PCA, with the anti-CD3/CD28 stimulated samples forming a distinct cluster, whereas IL-1β and media treated samples clustered together (Fig. 4B). Within the branch containing IL-1β treated cells and media treated cells, the samples form subclusters based on the blood donor. Finally, the samples were further divided based on cell surface phenotype, that is IL-1RI$^+$ and IL-1RI$^-$ cells. Therefore, for both the PCA and hierarchical clustering, the greatest effect

on the Th17 transcriptome was activation with anti-CD3/CD28, with lesser effects seen for IL-1β treatment or the expression of IL-1RI.

When comparing the media treated IL-1RI$^+$ and IL-1RI$^-$ Th17 cells to each other, differential expression analysis revealed 2844 genes whose expression differed with a p-adj ≤ 0.05 between the two populations (Fig. 4C). Consistent with the sorting strategy, IL-1 receptor genes *IL1R1* and *IL1R2* were among the most highly differentially expressed genes by both fold change (FC) and adjusted p-value. Several characteristic Th17 cell genes including *RORC, IL23R,* and *LGALS3* were upregulated in IL-1RI$^+$ Th17 cells (44). Gene expression for the regulatory T cell markers *FOXP3, LRRC32, IL2RA, and CTLA4* were also more highly expressed by these cells (45–47). This finding is consistent with previous reports that IL-1RI$^+$ cells include a population of IL-17 producing regulatory T cells (48, 49). TCR repertoire analysis revealed that IL-1RI$^+$ and IL-1RI$^-$ Th17 cells were very similar with respect to TCR usage. Both populations had diverse TCR alpha and beta repertoires with no restriction in terms of V or J usage (fig. S2A). Additionally, there was no preference for specific V-J region combinations (fig. S2B).

In IL-1RI$^+$ Th17 cells, IL-1β stimulation significantly induced the expression of 339 genes with a FC ≥ 1.5 and p-adj ≤ 0.05 compared to media treated cells (Fig. 4D). Functional analysis of these genes with Ingenuity Pathway Analysis (IPA) found that the most significantly enriched canonical pathway was “Th17 cell activation” ($p = 2.51 \times 10^{-17}$), with our dataset containing 21% of the pathway genes. (Table I). These genes encoded effector Th17 cytokines (*IL26, IL17A, IL23A, IL22*), transcription factors (*AHR, HIF1A, BATF*), and signaling molecules (*IRAK2, IRAK3, JAK3*). Of the 19 genes, 15 were more strongly induced by anti-CD3/CD28 vs IL-1β (14/15 were significant, p-adj ≤ 0.05), including *IL17A, IL17F,* and *IL22,* whose expression is increased by approximately 100-fold, 50-fold, and 25-fold, respectively (Table I). Overall, anti-CD3/CD28 induced greater expression of 211 of the 339 genes compared to IL-1β treatment.

Aside from the ‘Th17 activation pathway’ genes, the transcript most induced by IL-1β in the 320 remaining genes was *CXCL8,* which encodes the protein IL-8 (Fig. 4D). The DESeq2 normalized counts for *CXCL8* were similar for cells treated with IL-1β or anti-CD3/CD28. The level of IL-8 protein secreted by memory CD4$^+$ T cells was roughly equal after stimulation with IL-1β or anti-CD3/CD28, reflecting the similar mRNA expression in IL-1RI$^+$ Th17 cells (Figs. 4D, 4E). Although IL-26 protein was induced 2-fold greater by anti-CD3/CD28 vs IL-1β treatment (Fig. 3D), the *IL26* mRNA induction was 7-fold greater (Fig. 4D). In contrast, IL-17A, IL-17F, and IL-22 proteins were detected following anti-CD3/CD28 but not IL-1β treatment (Fig. 2C, Fig. 3E); as stated, the corresponding mRNAs were induced by greater than 25 fold following anti-CD3/CD28 vs. IL-1β treatment (Fig. 4D). This comparison of mRNA transcript upregulation to protein secretion suggests a threshold for protein induction such that the magnitude of mRNA induction by IL-1β is sufficient to produce IL-26 and IL-8 protein but not IL-17A, IL-17F, and IL-22.

We found using the gene set enrichment analysis tool Enrichr (50, 51) that genes upregulated by IL-1β in IL-1RI$^+$ Th17 cells with a p-adj ≤ 0.05 and a FC ≥ 1.5 were highly significantly enriched for RelA target genes ($p\text{-adj} = 2.7\times10^{-31}$) (Fig. 5A). This is consistent

with the canonical IL-1 signaling pathway, in which IL-1 receptor engagement activates MyD88, with subsequent activation of interleukin-1 receptor associated kinases and TNF-receptor associated factor proteins that activate NF-κB phosphorylation and translocation to the nucleus (52). The transcription factor with the second most significant target gene enrichment in this data set was MYB (p-adj = 3.9×10^{-8}). In contrast, Enrichr analysis of the upregulated genes in anti-CD3/CD28 treated IL-1RI^{+} Th17 cells revealed enrichment for genes regulated by a variety of transcription factors, including *RELA* (p-adj = 9.5×10^{-22}), *ETS1* (p-adj = 7.5×10^{-20}), *VDR* (p-adj = 5.3×10^{-17}), *GABP* (p-adj = 7.5×10^{-15}), and *FOXP3* (p-adj = 1.8×10^{-13}). These data suggest that IL-1β is sufficient to elicit an NF-κB regulated gene expression pattern that leads to IL-26 secretion but, unlike TCR activation, does not robustly stimulate activation of downstream genes under the control of a broad variety of transcription factors.

Given that both IL-1β and TCR stimulation of the IL-1RI^{+} Th17 cells led to induction of an NF-κB transcriptional signature and IL-26 secretion, and for IL-1β treatment the NF-κB downstream gene signature was dominant, we hypothesized that NF-κB played an important regulatory role in IL-26 expression. To test this, we inhibited NF-κB activation with the IκBα phosphorylation inhibitor Bay 11–7082 before activating memory CD4^{+} T cells with IL-1β or anti-CD3/CD28 antibodies (53, 54). We found that Bay 11–7082 completely abrogated IL-26 secretion from the cells (Fig. 5C). Therefore, we conclude that NF-κB activation is necessary for IL-26 production from T cells.

IL-1β stimulated T cells kill bacteria in an IL-26 dependent manner

The ability of Th17 cells to mount an IL-26 dependent antimicrobial response after TCR activation has been previously demonstrated (9, 55), so we thought it important to establish whether activation of memory CD4^{+} T cells through the alternative IL-1β pathway would lead to a functional antimicrobial response. Memory CD4^{+} T cells were cultured for 48 hours with or without 20 ng/ml IL-1β and IL-26 secretion in the supernatant was confirmed by ELISA (Fig. 6A). We found that T cell supernatants from IL-1β treated memory CD4^{+} T cells were directly antimicrobial against *E. coli,* leading to a 69% reduction in CFU formation compared to supernatants from untreated T cells (Fig. 6B). Incubating the supernatants with anti-IL-26 antibodies for one hour prior to the addition of bacteria reduced the antimicrobial ability of the supernatant by 56% while incubation of supernatants with isotype matched control antibodies did not. We found that recombinant IL-1β itself had no effect on bacterial viability (fig. S3). Bacteria incubated in unconditioned culture media was used as a negative control while incubation in media containing an antibiotic mixture of penicillin (50 U/ml) and streptomycin (50 μg/ml) was used as a positive control. These results establish that IL-1β can activate memory CD4^{+} T cells to mount an antimicrobial response that is mediated by IL-26 secretion.

Discussion

Given that Th17 cells produce the antimicrobial protein IL-26 that contributes to host defense against bacterial infection, we studied the immune pathways that result in the release of this cytokine in response to the pathogen. Here we establish that *M. leprae* can stimulate

memory CD4+ T cells to produce IL-26, but through distinct pathways with different kinetics. *M. leprae* and defined PRR ligands induced rapid and IL-1β dependent IL-26 secretion from PBMCs as early as 3 hours. IL-1β stimulated the secretion of IL-26 from memory $CD4^+$ T cells independent of and more rapidly than by TCR activation. IL-1β did not induce other Th17 cytokines, unlike TCR activation with anti-CD3/CD28 antibody complexes. We identified a subpopulation of Th17 cells that express IL-1RI and are responsible for virtually all of the IL-26 production by Th17 cells in response to either IL-1β or TCR activation. Analysis of the transcriptome of IL-$1RI^+$ Th17 cells indicated IL-1β treatment induced characteristic Th17 genes and revealed that IL-1β also stimulates IL-8 release from IL-$1RI^+$ Th17 cells. IL-1β upregulated an overall gene expression pattern regulated by NF-κB, whereas genes induced by TCR stimulation were regulated by multiple transcription factors. We established that NF-κB regulates IL-26 production in T cells using NF-κB activation inhibitors. Furthermore, IL-1β activated memory $CD4^+$ T cells exhibited antimicrobial activity against *E. coli*, which was blocked by anti-IL-26 neutralizing antibodies. Taken together, these results identify “antimicrobial Th17 cells” that can contribute to innate immunity by their expression of IL-1RI and very rapid response to IL-1β resulting in IL-26 dependent killing of bacteria.

Previously, Th17 cells were shown to secrete IL-26 following activation via the TCR as part of the adaptive immune response (9, 11). We elucidate a novel IL-1 dependent pathway for IL-26 secretion from memory IL-$1RI^+$ Th17 cells that is independent of and more rapid than TCR activation as part of the innate immune response. The rapidity of the IL-1β pathways compared to TCR triggering enables Th17 cells at the site of infection to quickly produce an antimicrobial protein even before the response to antigen occurs. Furthermore, we demonstrate that the ability of IL-1β to trigger IL-26 results in a direct antimicrobial activity against a common mucosal pathogen *E. coli*, and that the addition of anti-IL-26 antibodies blocked killing of the bacteria. This did not preclude other antimicrobial mediators, for example we demonstrate that IL-1β induces IL-$1RI^+$ Th17 cells to secrete IL-8, which contains an antimicrobial peptide released following acid hydrolysis (56). These data challenge the current paradigm for how Th17 cells respond to infection by demonstrating that human IL-$1RI^+$ Th17 cells can respond rapidly by an IL-1β mediated pathway prior to activation of their known TCR pathway. Thus, we describe a novel T cell subpopulation that can take advantage of both innate and adaptive activation pathways to participate in host defense.

We demonstrate depletion of IL-$1RI^+$ memory $CD4^+$ T cells abrogated the IL-1β induced IL-26 response and that IL-1β stimulates IL-26 secretion from Th17 cells but not from Th1 and Th2 cells. However, we cannot preclude that other IL-1RI expressing T cell populations have the capacity to secrete IL-26 in response to IL-1β. In addition, there is evidence that non-lymphoid cells including alveolar macrophages, fibroblast-like synoviocytes, and vascular smooth muscle cells are capable of producing IL-26 (16, 17, 57). It is therefore possible that cells of the myeloid lineage such as monocytes and dendritic cells contribute directly to the higher level of IL-26 production in IL-1β treated PBMC vs. memory $CD4^+$ T cells. These myeloid cells may also contribute to the production of cytokines which amplify IL-26 secretion. For example, a combination of IL-1β and TNF-alpha enhances IL-26 secretion in smooth muscle cells (17).

Our data indicate that IL-1β activation of Th17 cells induced the release of IL-26 and IL-8. The ability of lymphocytes to rapidly respond to exogenous cytokines to produce Th17 cytokines has been observed for other unique lymphoid subpopulations. For example, IL-23 and IL-1β have been shown to induce IL-17 secretion from γδ T cells and IL-17 and IL-22 secretion from ILC3 cells (19–21). However, γδ T cells express a restricted range of TCRs and ILCs lack TCRs altogether, precluding their participation in classic memory responses. Analysis of T cell receptor genes in our RNA sequencing data demonstrated that IL-1RI$^+$ Th17 cells expressed a wide range of conventional αβ T cell receptor genes. Our data is distinct in demonstrating that IL-1β triggers conventional αβ T cells to secrete IL-26 and mount an antimicrobial response independent of TCR activation.

IL-1β treatment of memory CD4$^+$ T cells was characterized by the secretion of IL-26 and the predominant induction of NF-κB pathways, whereas in addition, TCR activation triggered the full spectrum of Th17 cytokines and a diverse array of signaling pathways. We demonstrated that IL-1β induction of IL-26 protein secretion was indeed dependent on NF-κB signaling. The IL-26 gene is found on chromosome 12q15 and is flanked by IL-22 and IFN-γ, with this gene order conserved across vertebrates (58). In agreement with our data, it has been shown that an NF-κB binding site within this locus is involved in the distinct regulation of the IL-26 gene (58). It is known that IL-1 signals through MyD88, leading to induction of genes downstream of NF-κB. Activation of Toll-like receptors also proceeds through the same MyD88—NF-κB signaling pathway (52, 59). Thus, IL-1RI$^+$ Th17 cells can take advantage of an archetypal innate signaling pathway to mount a rapid antimicrobial response.

Th17 cells have been previously described as “pathogenic” vs. “non-pathogenic” based on their contribution to tissue injury and their secreted cytokine pattern; pathogenic Th17 cells produce IL-17A and IFN-γ whereas non-pathogenic Th17 cells produce IL-17A and IL-10 (60). We define “antimicrobial Th17 cells” based upon the expression of IL-1RI, production of IL-26 and the ability to mediate a rapid antimicrobial response against extracellular bacteria. In addition to their ability to release the antimicrobial protein IL-26, our transcriptome analysis revealed that the IL-1RI$^+$ Th17 cells express Treg markers *FOXP3, LRRC32*, *CTLA4* and *CD25.* It is thought that some of these cells represent intermediates in Th17 development from Treg precursors as they secrete IL-17A, express *FOXP3*, suppress T cell proliferation, and exhibit a central memory phenotype (48, 49). Alternatively, CD25 and CTLA4 are features of activated T cells and IL-1RI has been shown to be upregulated by TCR activation, therefore it is possible that IL-1RI$^+$ Th17 isolated *ex vivo* may represent a recently activated group of cells (61, 62). In this context, IL-1β could trigger a rapid response in IL-1RI$^+$ Th17 cells as well as prolong the antimicrobial response within the slower adaptive immune setting. We note that activation of Th17 cells via the TCR but not IL-1β induces other cytokines such as IL-17A and IL-22, which act to trigger the release of other antimicrobial peptides from bystander cells, perhaps augmenting the effect of IL-26. Nonetheless, we find that IL-1RI$^+$ Th17 cells were the predominant producers of IL-17A in response to anti-CD3/CD28, in agreement with a previous report that also showed and that IL-1β augments cytokine responses from these cells (28).

This work establishes the unusual role of a novel subset of Th17 T cells in contributing to both innate and acquired immunity in the response to microbial infection. Th17 cells have long been linked to host defense against extracellular bacteria, but more recently Th17 cells have been shown to have a role against intracellular bacteria including *M. leprae* (63, 64). IL-26 specifically has also been shown to contribute to host defense in leprosy by entering *M. leprae* infected macrophages and reducing of the viability of the intracellular bacteria (65). The identification of the factors that regulate the “antimicrobial Th17” compartment and IL-26 provides potential new targets for augmenting the immune response to bacterial infection in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

The following abbreviations are used in this work:

ILC	Innate lymphoid cell
PRR	Pattern recognition receptor
IL-1RI	Interleukin-1 receptor 1

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Key Points

1. TLR ligands induce IL-26 secretion from PBMCs in an IL-1β dependent manner.
2. IL-1β induces rapid IL-26 release by IL-1RI^{+} Th17 cells without TCR activation.
3. IL-1β stimulated T cells mediate an antimicrobial activity via IL-26.

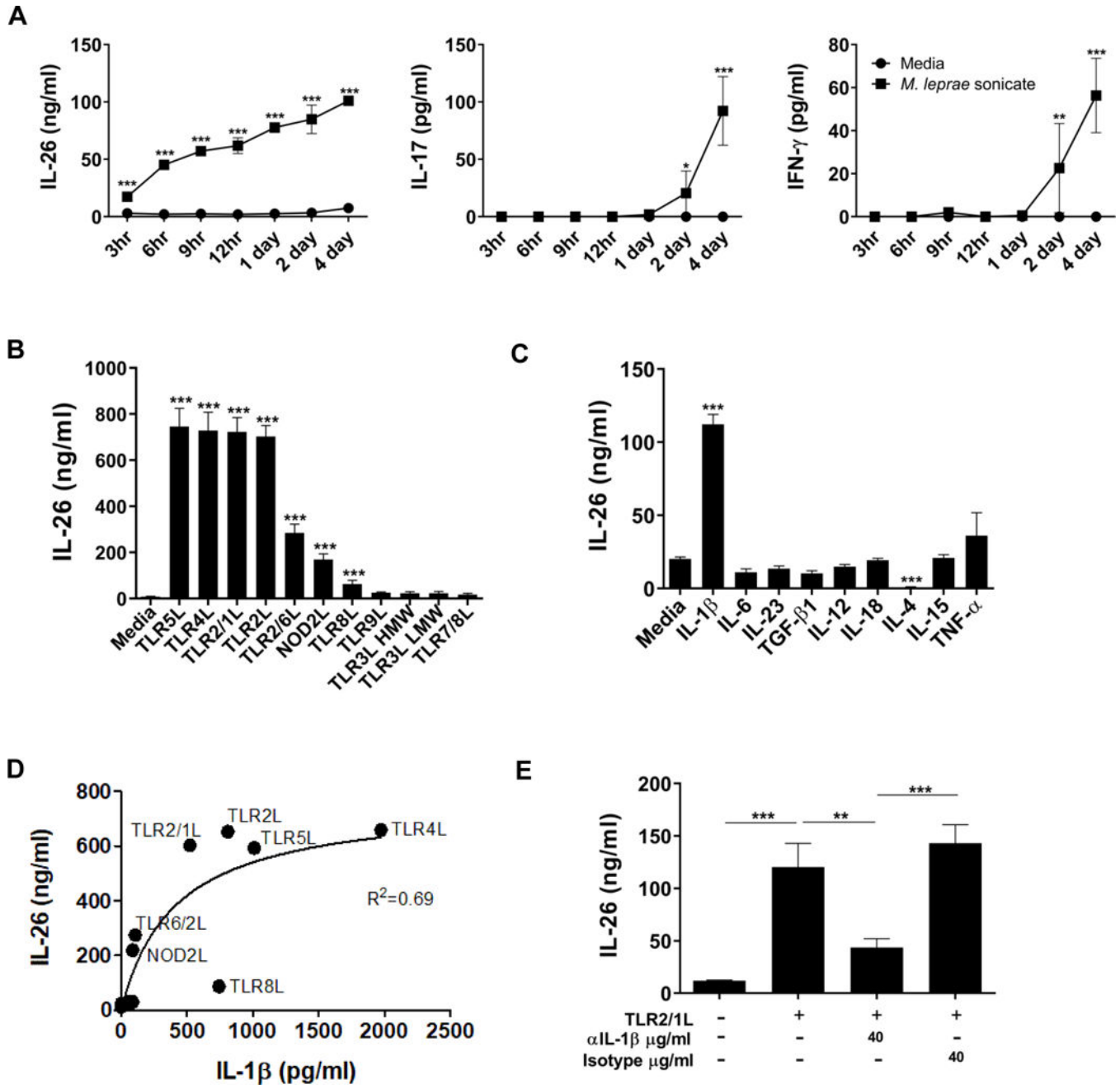

Figure 1. PBMCs rapidly secrete IL-26 in response to innate ligands in an IL-1β dependent manner.

(**A**) PBMCs derived from tuberculoid leprosy patients were incubated or not with 10 μg/ml *M. leprae* sonicate for the indicated amount of time before cell free supernatants were collected and cytokine concentrations measured by ELISA, n = 3. (**B**) PBMCs were treated with the indicated PRR ligands for 16 hours before IL-26 concentration in cell free supernatants was measured by ELISA, n = 6. (**C**) PBMCs were treated with 20 ng/ml of the indicated cytokine for 48 hours before IL-26 concentration was measured in cell free supernatants by ELISA, n = 3. (**D**) The average IL-1β vs IL-26 concentration in supernatants

of PRR ligand stimulated PBMCs was plotted with GraphPad Prism 8.0 software and a sigmoidal dose-response curve was fitted to the data, $y= (761.8*x^{1.002})/(416.6+x^{1.002})$, $R^2 = 0.69$. (**E**) PBMCs were stimulated with 1 ng/ml of the TLR2/1 agonist Pam3CSK4 overnight in the presence or absence of blocking anti-IL-1β antibody or isotype matched control antibody, n = 4. (**A-C, E)** Data are represented as mean ± SEM, statistics calculated using two-way ANOVA with Sidak's post-test (A) or one-way ANOVA with Tukey's post-test (B, C, E). *$*p \leq 0.05$. $**p \leq 0.01$. $*** p \leq 0.001$*

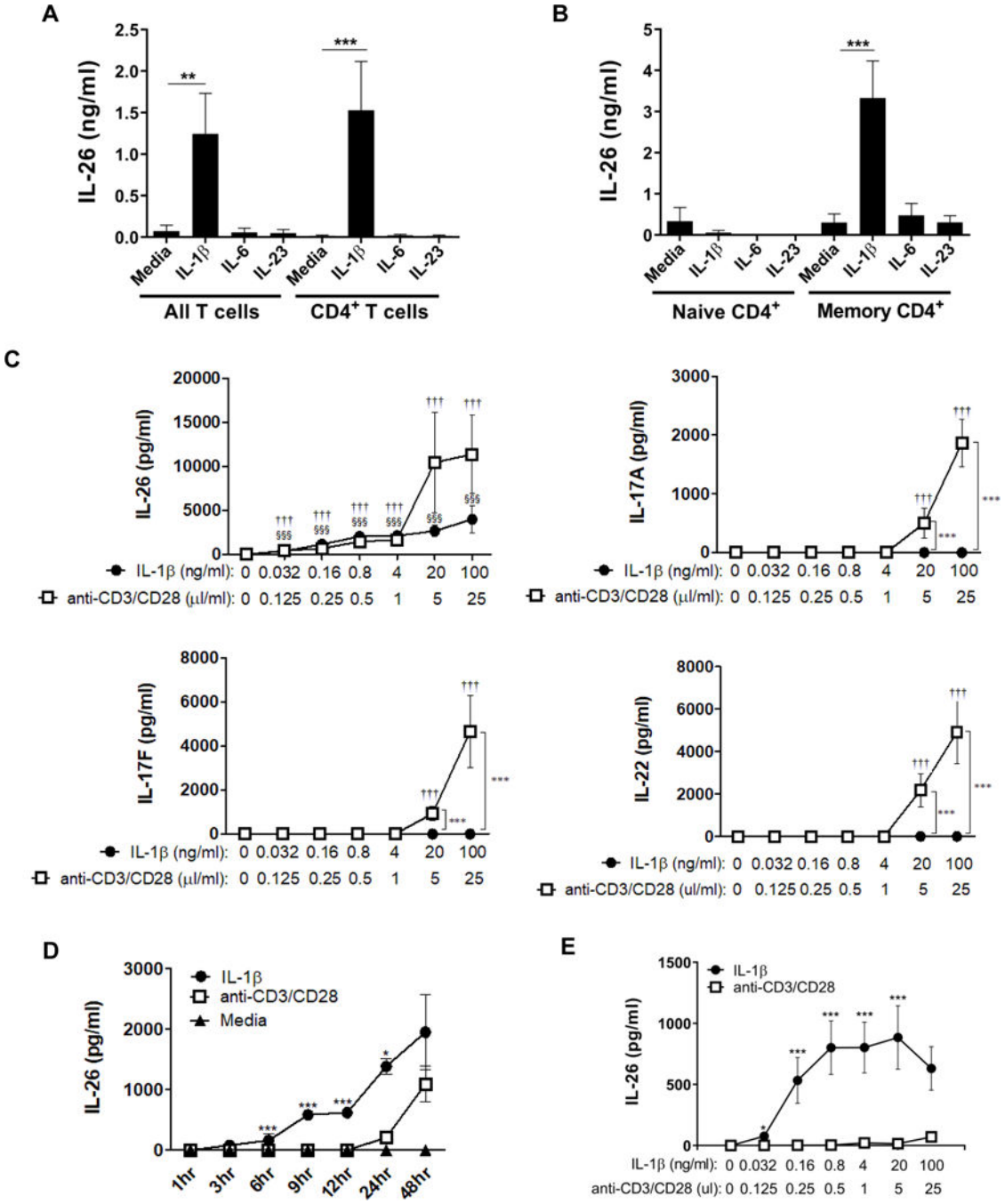

Figure 2. IL-1β induces rapid IL-26 secretion from memory CD4+ T cells.

(A-B) Immunomagnetically selected T cell populations were incubated with 20 ng/ml of the indicated cytokine for 48 hours before cell free supernatants were analyzed by ELISA, n = 8 (A) n = 3 (B). (**C**) Memory $CD4^+$ T cells were incubated with the indicated amounts of IL-1β or anti-CD3/CD28 tetrameric antibody complexes for 48 hours before cell free supernatants were analyzed for IL-26, IL-17A, IL-17F, and IL-22 concentrations by ELISA, n = 4. Data are represented as mean ± SEM, statistics calculated using one-way ANOVA with Tukey's post-test. Levels of significance for three comparisons are indicated as follows:

untreated cells to IL-1β treated cells, §; Untreated cells to anti-CD3/CD28 treated cells, †; IL-1β treated cells to anti-CD3/CD28 treated cells, *. (**D**) Memory $CD4^+$ T cells were incubated or not with 20 ng/ml IL-1β or 0.5 μl/ml anti-CD3/CD28 tetrameric antibody complexes and cell free supernatants were analyzed for IL-26 secretion at the indicated time points, n = 3. (**E**) Memory $CD4^+$ T cells were stimulated with the indicated amount of IL-1β or anti-CD3/CD28 for 4 hours before IL-26 concentrations in cell free supernatants were analyzed by ELISA, n = 3. (**A-E**) Data are represented as mean ± SEM, statistics calculated using one-way ANOVA with Tukey's post-test (A, B, C, E) or two-way ANOVA with Tukey's post-test (D). Asterisks denote significance between IL-1β and anti-CD3/CD28 treatment (C, D, E). *$*p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$*

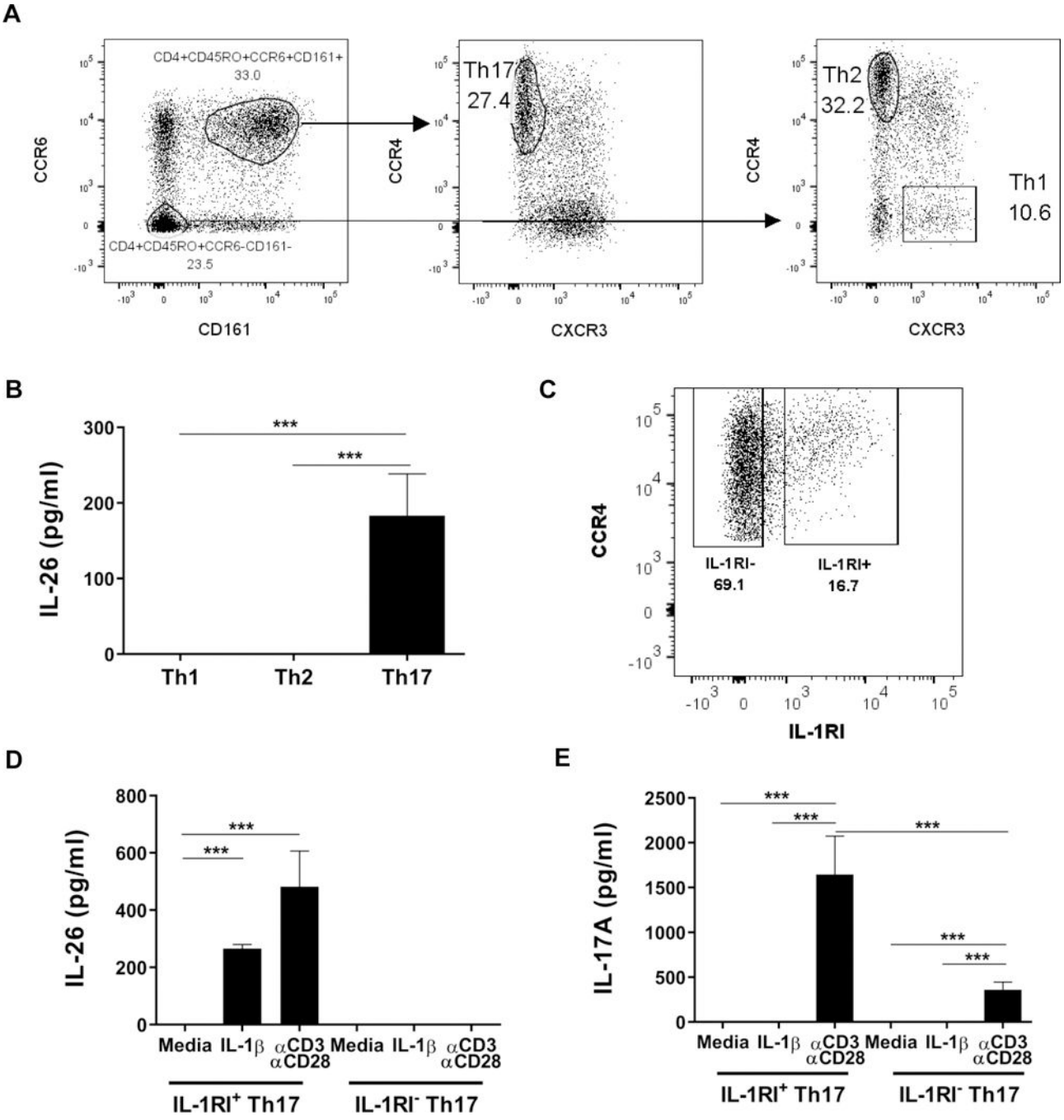

Figure 3. IL-1RI+ Th17 cells secrete IL-26 in response to IL-1β.

(**A**) Sorting scheme used to isolate helper T cell subsets, a representative donor is shown, n = 3. (**B**) Isolated Th1, Th2, and Th17 cells were stimulated with IL-1β for 48 hours and cell free supernatants were analyzed for IL-26 secretion by ELISA, n = 3. (**C**) A representative sorting scheme for isolating IL-1RI^{+} and IL-1RI^{-} Th17 cells, n= 3. (**D, E**) IL-1RI^{+} and IL-1RI^{-} Th17 cells were stimulated with 100 ng/ml IL-1β or 5 μl/ml anti-CD3/CD28 for 9 hours before IL-26 (D) and IL-17A (E) was measured in cell free supernatants by ELISA, n

= 3. (**B,D**) Data are represented as mean ± SEM, statistics calculated using one-way ANOVA with Tukey's post-test (B, D, E). ***$p \leq 0.01$. *** $p \leq 0.001$*

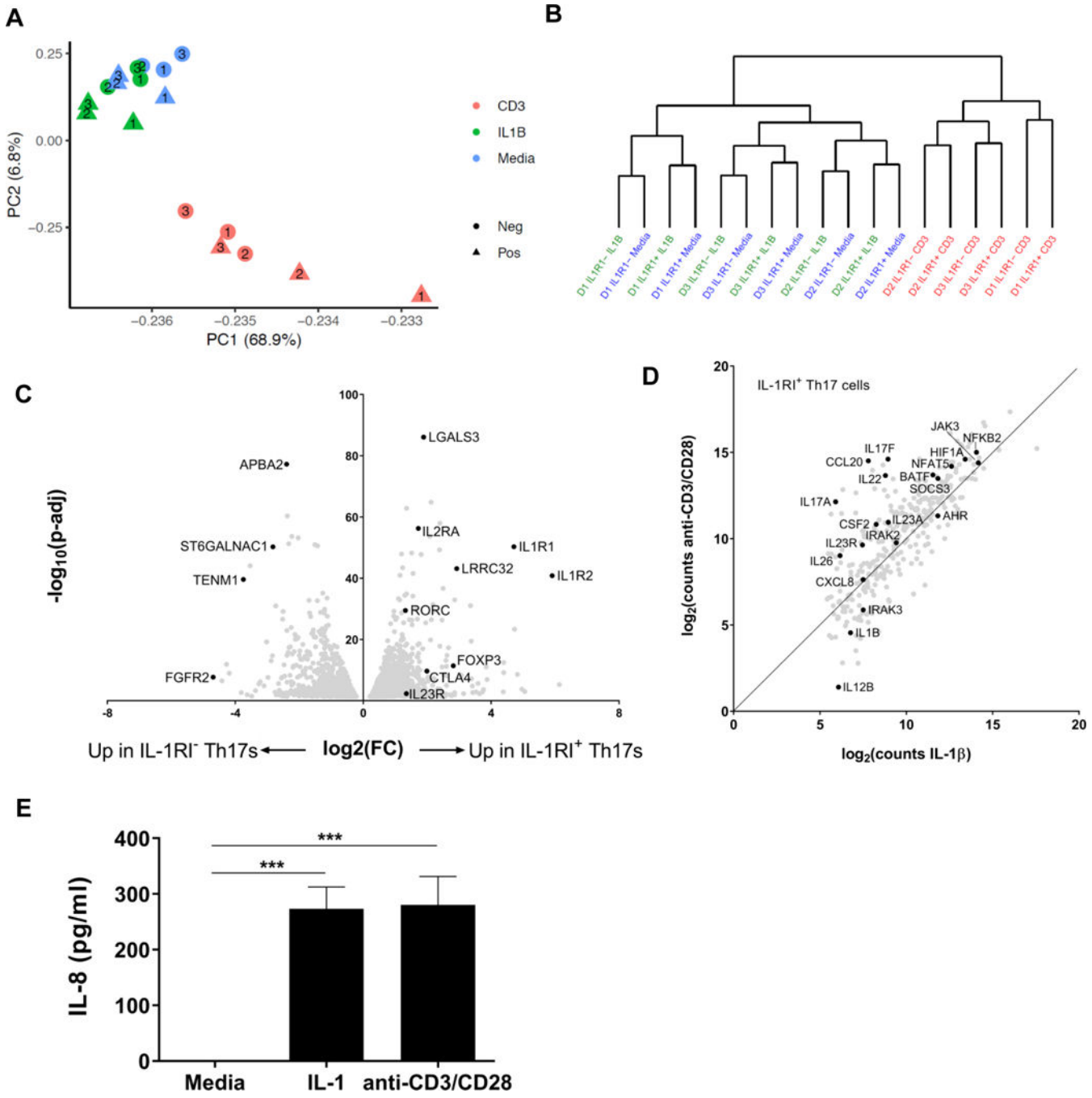

Figure 4. IL-1β upregulates Th17 gene expression program in IL-1RI$^+$ Th17 cells.

(**A**) Unsupervised principal component analysis of IL-1RI$^+$ and IL-1RI$^-$ Th17 cells from 3 donors treated with IL-1β, anti-CD3/CD28, or media as a control. Numbers within symbols indicate the donor. (**B**) Hierarchical clustering of the samples. (**C**) Differential gene expression in untreated IL-1RI$^+$ Th17 cells vs. IL-1RI$^-$ Th17 cells is represented in a volcano plot and some of the most differentially expressed genes are named and highlighted with black dots. (**D**) Genes with a (FC) ≥ 1.5 and p-adj ≤0.05 after treatment of IL-1RI$^+$ Th17 cells with IL-1β vs media are plotted based on their normalized counts after IL-1β or

anti-CD3/CD28 treatment. The line y = x was plotted on the graph to show genes with greater relative counts in each condition. Genes in the IPA "Th17 cell activation" canonical pathway and *CXCL8* are highlighted with black dots. (**E)** Memory $CD4^+$ T cells were treated with 100 ng/ml IL-1β and 5 μl/ml anti-CD3/CD28 for 48 hours before supernatants were collected and IL-8 protein concentrations were measured by ELISA, n = 4. Data are represented as mean ± SEM, statistics calculated using one-way ANOVA with Tukey's post-test. **** $p \leq 0.001$.*

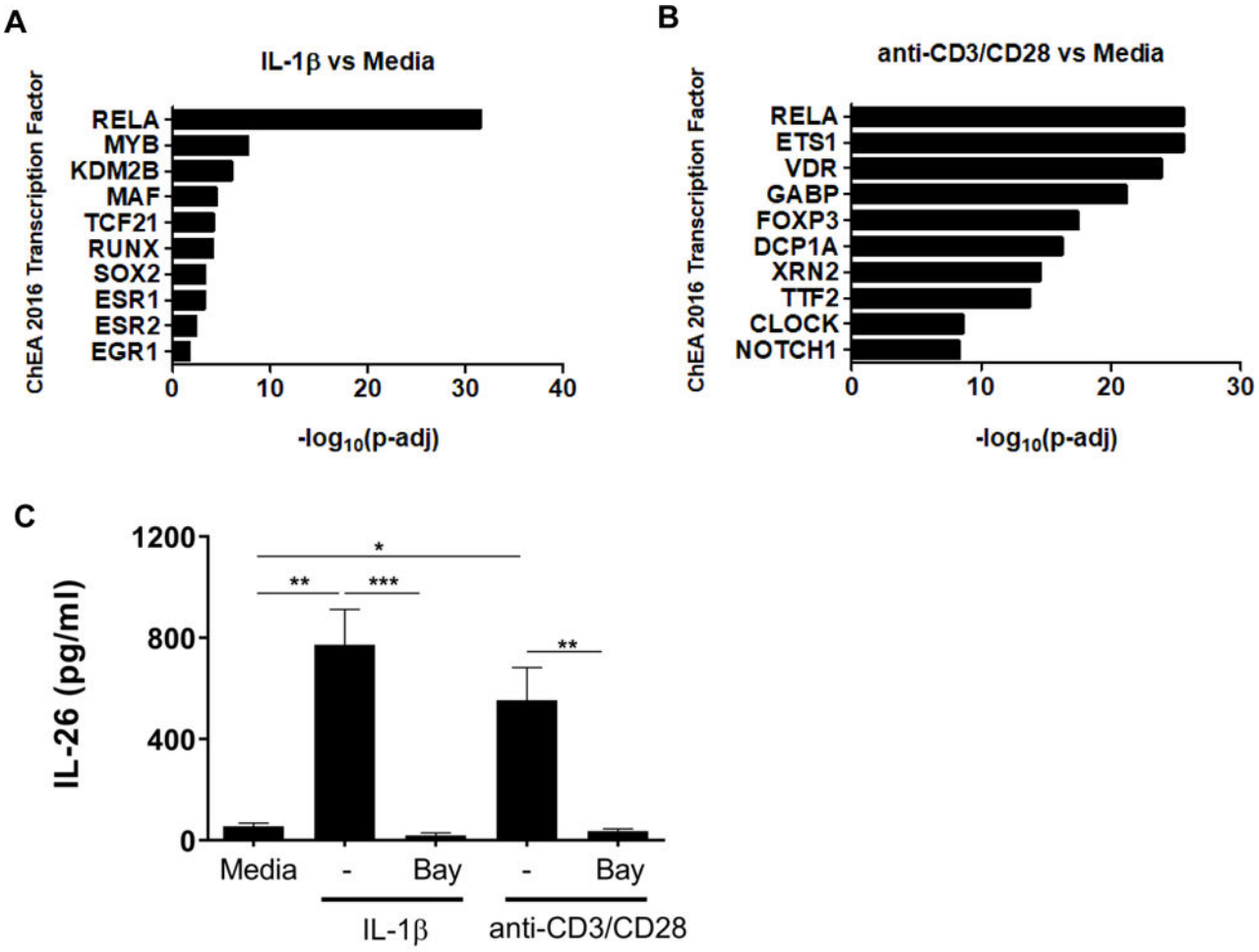

Figure 5. Upstream analysis of RNA-seq dataset reveals IL-26 dependence upon NF-κB.

(**A, B**) Genes with a $\log_2(FC) \geq 0.58$ and p-adj ≤0.05 after treatment of IL-1RI$^+$ Th17 cells with IL-1β (A) or anti-CD3/CD28 (B) were analyzed for enrichment of upstream transcription factors using Enrichr and plotted with their -$\log_{10}$(p-adj). **C.** Memory CD4$^+$ T cells were stimulated with either 20 ng/ml IL-1β or 5 μl/ml anti-CD3/CD28 for 24 hours, with or without 1 hour pretreatment with 10μM Bay 11–7082, an NF-κB activation inhibitor. IL-26 secretion into cell supernatants was measured by ELISA, n = 3. Data are represented as mean ± SEM, statistics calculated using one-way ANOVA with Tukey's post-test. **p ≤ 0.05, *** p ≤ 0.001.*

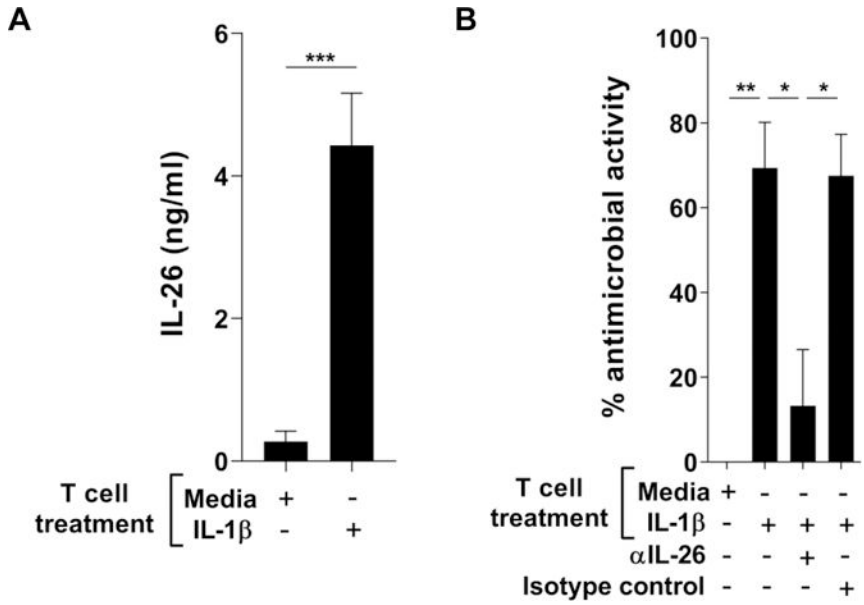

Figure 6. IL-1β stimulated T cell supernatants are antimicrobial.

(**A**) IL-26 was measured by ELISA in cell free supernatants collected from memory $CD4^+$ T cells that were stimulated or not with 20 ng/ml IL-1β for 48 hours. **B.** *E. coli* was inoculated into cell culture supernatants from 48 hour IL-1β or media control stimulated T cells, some pre-incubated with 20 μg/ml anti-IL-26 blocking antibody or isotype matched control antibody, and then incubated overnight at 37°C with shaking at 250 rpm. Percent antimicrobial activity was determined by CFU assay of a dilution series plated on LB agar plates, n = 3. **(A, B)** Data are represented as mean ± SEM, statistics calculated using paired Student's t-test (A) or one-way ANOVA with Tukey's post-test (B). *$p \leq 0.05$. **$p \leq 0.01$.

Table I.

“Th17 cell activation” pathway genes significantly upregulated by IL-1β treatment of IL-1RI$^+$ Th17 cells.

The IPA canonical pathway “Th17 cell activation” was the most significantly enriched canonical pathway within our dataset ($p = 2.51 \times 10^{-17}$). This pathway contained 90 genes, 19 of which, or 21%, were contained in our dataset and are displayed in the table.

Gene	DESeq2 FC IL-1β vs media	p-adj IL-1β vs media	DESeq2 FC IL-anti-CD3 CD28 vs IL-1β	p-adj anti-CD3/CD28 vs. IL-1β
AHR	1.6	1.2×10^{-9}	0.8	9.8×10^{-3}
BATF	2.1	4.6×10^{-32}	4.7	4.4×10^{-29}
CCL20	1.6	6.6×10^{-3}	95.8	4.5×10^{-44}
CSF2	25.1	8.4×10^{-5}	12.6	2.5×10^{-4}
HIF1A	1.7	2.8×10^{-15}	2.4	1.3×10^{-17}
IL1B	70.3	3.1×10^{-13}	0.3	1.1×10^{-4}
IL12B	145.6	1.6×10^{-7}	0.01	9.5×10^{-3}
IL17A	5.3	3.2×10^{-4}	105.5	4.2×10^{-13}
IL17F	27.2	3.5×10^{-52}	49.2	2.7×10^{-190}
IL22	21.3	6.8×10^{-50}	25.9	2.5×10^{-7}
IL23A	1.6	1.0×10^{-4}	4.2	1.4×10^{-37}
IL23R	1.8	4.4×10^{-3}	4.8	9.7×10^{-34}
IL26	2.4	1.4×10^{-2}	7.3	2.5×10^{-4}
IRAK2	3.5	1.1×10^{-21}	1.3	2.0×10^{-2}
IRAK3	3.4	3.5×10^{-9}	0.3	1.8×10^{-6}
JAK3	1.8	4.9×10^{-5}	1.2	1.8×10^{-1}
NFAT5	1.5	1.4×10^{-7}	3.1	1.2×10^{-23}
NFKB2	4.5	2.5×10^{-133}	2.0	1.7×10^{-17}
SOCS3	2.3	1.3×10^{-2}	3.6	7.2×10^{-16}