IL-32 Is a Host Protective Cytokine against *Mycobacterium tuberculosis* in Differentiated THP-1 Human Macrophages

Xiyuan Bai, Soo-Hyun Kim, Tania Azam, Mischa T. McGibney, Hua Huang, Charles A. Dinarello and Edward D. Chan

*J Immunol* 2010; 184:3830-3840; Prepublished online 26 February 2010; doi: 10.4049/jimmunol.0901913

http://www.jimmunol.org/content/184/7/3830

**References**

This article cites 85 articles, 49 of which you can access for free at:
http://www.jimmunol.org/content/184/7/3830.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-32 Is a Host Protective Cytokine against Mycobacterium tuberculosis in Differentiated THP-1 Human Macrophages

Xiyuan Bai,* Soo-Hyun Kim,† Tania Azam,‡ Mischa T. McGibney,* Hua Huang,§ Charles A. Dinarello,‡ and Edward D. Chan*†¶

Macrophages provide a first line of defense against Mycobacterium tuberculosis. However, in instances where macrophage activation for killing is suboptimal, M. tuberculosis is capable of surviving intracellularly. IL-32 is a recently described cytokine induced by M. tuberculosis in a variety of cell types including human monocytes and macrophages. In this study, we investigated the biological significance of IL-32 in an in vitro model of M. tuberculosis infection in differentiated THP-1 human macrophages in which IL-32 expression was silenced using stable expression of short hairpin RNA (shRNA). Inhibition of endogenous IL-32 production in THP-1 cells that express one of three distinct shRNA-IL-32 constructs significantly decreased expression was silenced using stable expression of short hairpin RNA (shRNA). Inhibition of endogenous IL-32 production in THP-1 cells compared with THP-1 cells stably expressing a scrambled shRNA. In THP-1 cells infected with M. tuberculosis and stimulated with rIL-32, a greater level of apoptosis was observed compared with that with M. tuberculosis infection alone. Obversely, there was significant abrogation of apoptosis induced by M. tuberculosis and a concomitant decrease in caspase-3 activation in cells depleted of endogenous IL-32. rIL-32γ significantly reduced the number of viable intracellular M. tuberculosis bacteria, which was modestly but significantly abrogated with a caspase-3 inhibitor. We conclude that IL-32 plays a host defense role against M. tuberculosis in differentiated THP-1 human macrophages. The Journal of Immunology, 2010, 184: 3830–3840.

Tuberculosis (TB) remains a leading cause of suffering and death worldwide (1). Although alveolar macrophages provide a first line of defense against Mycobacterium tuberculosis, macrophages may also provide a safe intracellular niche for mycobacteria if host cells are unable to kill them (2, 3). Whether the initial infection leads to complete elimination, a progressive infection, or a state of latent infection ultimately depends on the effectiveness of both the innate immune system and the cell-mediated immune system, orchestrated by macrophages and dendritic cells. Presentation of mycobacterial Ags by macrophages and dendritic cells to lymphocytes in regional lymph nodes, resulting in the differentiation, activation, and efflux of effector T cells to sites of infection is a major pillar in the effective host response against M. tuberculosis. Known killing mechanisms against intracellular M. tuberculosis include phagosome-lysosome fusion, apoptosis, autophagy, direct toxic effect of NO, and antimicrobial peptides, such as cathelicidin, defensins, and granulysin (4–15).

The collaboration between APCs and T cells culminating in their mutual activation and participation in the granulomatous response are mediated in large part by the release of cytokines, chemokines, and other effector molecules by both phagocytes and T cells. Cytokines important in host defense against M. tuberculosis and other pathogenic mycobacteria in both experimental animals and humans include TNF-α, IFN-γ, IL-8, IL-12, IL-17, IL-18, and IL-23 (16–27). By contrast, overexpression of Th2 or Th2-like cytokines, such as IL-4, IL-10, and TGF-β, is generally considered to predispose the host to M. tuberculosis (28–34), although controversies exist (35–37). Categorizing a cytokine as being helpful or harmful to the host in the context of TB is likely an oversimplistic view. For example, although TNF-α is critical in maintaining granuloma formation and containing mycobacterial infections, in the presence of IL-4, TNF-α is also responsible for many of the deleterious clinical manifestations, such as fever, tissue necrosis, anorexia, and weight loss (29, 38). Similarly, IL-4, IL-10, and TGF-β may be necessary to the host in the latter parts of the host immune response to prevent relentless inflammation and limit tissue damage. More recently, with mice with genetic disruption of IL-27R, IL-27 was shown to have the seemingly paradoxical effect of increasing M. tuberculosis burden and yet reducing cellular inflammation and lung pathology, resulting in a net effect of increased survival in the infected mice (39, 40).

IL-32 is a recently described cytokine produced by T lymphocytes, NK cells, epithelial cells, endothelial cells, monocytes, macrophages, and dendritic cells (41–45). IL-32 is also produced by human pancreatic periacinar myofibroblasts, pancreatic ductal cells, synovial fibroblasts, and marrow stromal cells in patients with myelodysplastic syndrome (46–49). IL-32 has proinflammatory properties in that it can induce production of TNF-α, MIP-2, IL-6, and IL-8 (43, 50). In turn, cytokines such as IFN-γ, IL-1β, IL-12, and IL-18 can induce IL-32 production (43). Six different isoforms of IL-32 have been identified, namely, IL-32α, β, γ, δ, ε, and ζ (43, 51). Depending on the cell type and stimulus, different isoforms may predominate (44). IL-32γ is the most biologically active
isoform in inducing cytokine production, perhaps because this isoform has no exonic deletions (52).

Although the receptor for IL-32 has not been found, IL-32 can induce activation of the MAPK and NF-κB signaling pathways (43, 47, 53). Interestingly, during the search for the receptor for IL-32, it was found that proteinase 3, independent of its serine protease activity, binds to IL-32 and cleaves it into fragments that are more active, in terms of induction of cytokines, than the parent molecule (54, 55).

We previously showed that IL-32 is induced by \textit{M. tuberculosis} and that in PBMCs induction of IL-32 by \textit{M. tuberculosis} occurred in an IL-18- and IFN-γ-dependent mechanism (56). However, the biological significance of endogenous IL-32 in \textit{M. tuberculosis} infection remains unknown. Because IL-32 is induced by cytokines and itself induces cytokines that are of immense importance in the control of \textit{M. tuberculosis}, we hypothesized that IL-32 may play a host defense role against \textit{M. tuberculosis}. Because mouse homologue of IL-32 has not yet been found, murine models could not be used to study the role of IL-32 in host defense against \textit{M. tuberculosis}. Therefore, we used small interfering RNA (siRNA) technology to reduce the level of endogenous IL-32 to study its role in \textit{M. tuberculosis} infection of a human monocytic/macrophage cell line. We chose to create stable cell lines that express short hairpin RNA (shRNA) sequences. The human myeloid cell line THP-1 was used as a model for human macrophages in in vitro infection studies for two reasons. First, THP-1 cells are amenable to stable transfection with DNA fragments that encode shRNA sequences. Second, THP-1 cells have been shown to behave similarly to primary human macrophages in their immune responses to \textit{M. tuberculosis} (57, 58).

### Materials and Methods

#### Materials

The human promonocytic cell line THP-1 was obtained from the American Type Culture Collection (TIB-202, Manassas, VA). RPMI 1640 was obtained from Cambrex (East Rutherford, NJ). FBS was purchased from Atlanta Biologicals (Norcross, GA) and heat-inactivated at 56˚C for 1 h. THP-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine and were maintained at a concentration between 2 and 10 × 10^6 cells per milliliter. PMA was purchased from Sigma-Aldrich (St. Louis, MO). TRizol reagents, SuperScript II Reverse Transcriptase First-Strand cDNA, and PCR Platinum Taq DNA polymerase were purchased from Invitrogen Life Technologies (Carlsbad, CA). Apoptosis In Situ Detection Kit was purchased from Roche Diagnostics Systems (Somerville, NJ). 3L-32a and rL-32y proteins were expressed in \textit{Escherichia coli} as his-tagged-ligated N-terminal fusion proteins, followed by affinity purification on a TALON affinity column from Roche Diagnostics Systems. IL-10, and TGF-β were purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-human Bcl-2 Ab, Bax Ab, cytochrome c Ab, and Phototube-HRP Western Blot Detection System were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rIL-32a polyclonal Ab was produced by Rockland (Gilbertsville, PA) and purified according to the manufacturer’s protocol. All primers and oligonucleotide products were obtained from GeneLink (Hawthorne, NY).

#### Mycobacterial culture and reagents

The H37Rv strain of \textit{M. tuberculosis} was obtained from the American Type Culture Collection (27294). \textit{M. tuberculosis} was grown to late log phase in Middlebrook 7H9 supplemented with albumin dextrose complex. After incubation by rotation at 37˚C, aliquots were frozen at ~70˚C in 10°C McFarland standard stocks. Middlebrook 7H9, 7H10, and Middlebrook albumin dextrose complex enrichment were obtained from Difco (Detroit, MI).

#### Stable expression of shRNA–IL-32 sequences in THP-1 cells

To create stable clones of THP-1 cells that inhibit IL-32 protein expression by siRNA technology, three separate plasmids were generated in which sense and antisense sequences to the IL-32 gene are separated by a 9-bp spacer of a nonhomologous sequence (Table I). These DNA constructs were cloned downstream of the H1 promoter within the pSUPER.puro RNAi vector that also contains the \textit{PUROMYCIN-RESISTANT} gene (OligoEngine, Seattle, WA). The sense, antisense, and spacer DNA sequences encode shRNAs that contain antisense sequences to IL-32 mRNA (shRNA–IL-32). More specifically, the DNA sequences contain a 19-nt sense sequence of IL-32 cDNA, separated by a 9-mer spacer (TTCAGAGGA) from a 19-nt antisense sequence complementary to the sense sequence, followed by TTTTT as the transcriptional terminator. These three DNA sequences (Table I) were chosen from ten sequences provided by the RNAi Design Program from Dharmacon (Boulder, CO) based on the greatest homology to the three main isoforms of the IL-32 gene (α, β, and γ). Circular pSUPER.puro RNAi vector was linearized by BglII and HindIII restriction enzymes. The three chosen DNA sequences that contain sense and antisense sequences to IL-32 were ligated into the linearized pSUPER.puro RNAi vector. The insertion of the aforementioned DNA sequences was confirmed by DNA sequencing using the T7 primer prior to transfection into human monocytic THP-1 cells. In addition, a scrambled DNA sequence was also inserted into the pSUPER.puro RNAi vector to serve as a negative control.

The empty pSUPER.puro RNAi vector, one containing the scrambled DNA sequence, or each of the pSUPER.puro IL-32 siRNA plasmids were transfected into THP-1 cells by electroporation using the Cell Line Nucleofector Kit V (Amaxa, Gaithersburg, MD) according to the manufacturer’s instructions. We previously showed that IL-32 is induced by \textit{M. tuberculosis} was obtained from the American Type Culture Collection (27294). For a transfection efficiency of 80%, the mixture was transferred to an Amaxa certified cuvette. After the optimal nucleofector program for transfection by electroporation was used, the cuvette was rinsed with medium, and the cells were immediately transferred from the cuvette into the culture dish. Clones of THP-1 cells stably expressing shRNA–IL-32 were selected with 1 μg/ml puromycin for 12 d, followed by 0.5 μg/ml puromycin for an additional 12 d, with the medium changed every 3 d. Prior to experimentation, the THP-1 cells were treated with 15 ng/ml PMA overnight to allow differentiation into macrophages. 

#### RNA isolation and RT-PCR

After treatment of THP-1 monocytes/macrophages on six-well tissue culture plates, total RNA was extracted using the TRizol reagent, and cDNA was prepared using reverse transcriptase, according to the manufacturer’s instructions (Invitrogen Life Technologies). Briefly, 3 μg sample of total RNA, dissolved in diethyl pyrocarbonate-treated water, was heated at 65˚C for 10 min and then cooled on ice for 10 min. Then 1 μl of 10 mM dNTPs mix. 1 μl oligo(dT)12–18 primer, and enough diethyl pyrocarbonate-treated water were added to each sample for a total volume of 10 μl. After brief centrifugation, the samples were incubated at 65˚C for 5 min, incubated on ice for 1 min, and followed by addition of a reaction mixture that contained 2 μl 10× reverse transcriptase buffer, 4 μl 25 mM MgCl2, 2 μl 0.1 M DTT, and 1 μl RNaseOUT Recombinant Ribonuclease Inhibitor. The samples were mixed gently, collected by brief centrifugation, and then incubated at 42˚C for 2 min. One microliter (10 μl) of Superscript II Reverse Transcriptase was added (final continuous incubation at 42˚C for 50 min, and the reaction was terminated by heating at 70˚C for 15 min. The samples were treated with 1 μl RNase H to digest RNA at 37˚C for 30 min. The single-stranded cDNA products were used as templates in a 30 μl PCR amplification reaction according to the instructions of the PCR kit. Amplifications were done by initial denaturation at 94˚C for 4 min and 20 or 25 cycles of denaturation at 94˚C for 30 s, annealing at 60˚C for 45 s, extension at 72˚C for 1 min, and final extension at 72˚C for 4 min. The resulting PCR products were resolved by 1.5% agarose gel electrophoresis. The following primers were used: IL-32 (549 bp) sense, 5'-CTG AAG GCC CGA ATG CAC GAG C3'–antisense, 5'-GCA AAG GAT GTG TCA GTGTC3'; IL-32 (314 bp) sense, 5'-TGA GAG GCA GCA CCC AGA GC3’–antisense, 5’-CCGGT AACGTCGAGAAGAAGG3’–GAPHD sense, 5’-TCG ATG ACA CCT GTG TCA TGT GC3’; antisense, 5’-TCG CCG TGT TGA AGT CAG AGA G3’–Western blot

Briefly, THP-1 monocytes/macrophages were lysed using a phosphorosilane lysis buffer that contains 50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 2 mM NaVO4, 1 mM NaF, 1 mM PMSF, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. The cell lysates were vortexed, sonicated, and centrifuged at 10,000 × g for 10 min at 4˚C. Protein concentrations of the cell lysates were determined using the Bradford protein assay (Bio-Rad, Hercules, CA). The supernatants of the centrifuged lysates were mixed with equal volumes of 2× Laemmli sample buffer containing 4 μM DTT and boiled for 5 min. Twenty micrograms of protein for each condition were resolved by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, MA). After the membranes were
were blocked in 5% milk for 1 h. They were probed with Abs to IL-32 at a dilution of 1:500 (v/v), Bcl-2 (1:1000), Bax (1:1000), cytoskeleton c (1:1000), or β-actin (1:1000), followed by detection with HRP-conjugated anti-rabbit IgG in a 1:2000 dilution. Bands were visualized by chemiluminescence using the HRP Western Blot Detection System (Cell Signaling Technology) as described in the manufacturer’s protocol.

**In vitro infection of differentiated THP-1 human macrophages**

THP-1 cells were infected with *M. tuberculosis* H37Rv at a multiplicity of infection of ten bacilli to one macrophage. Briefly, THP-1 cells were seeded in 6- or 24-well flat-bottom tissue culture plates and allowed to adhere and differentiate at 37°C in humidified, 5% CO2 atmosphere for 24 h in the presence of 15 ng/ml PMA. After overnight incubation, the PMA-containing medium was replaced with fresh RPMI 1640 plus 10% FBS and 2 mM glutamine without PMA. For day 0 cells, the supernatants were collected and filtered after 1 h of infection. The cells were washed twice with a solution containing 50% RPMI 1640 and 50% 1X PBS, followed by lysis of the adherent cells using 500 μl 0.25% SDS solution in each well. After 5 min, the cells were viewed under the inverted microscope to confirm cell lysis. Then 500 μl 7H9 plating medium was added to each well. For day 4 and day 8 conditions, the cells were washed twice after 1 h of infection, and the medium was replaced with fresh RPMI 1640 containing 10% FBS and 2 mM glutamine. The cells were then incubated for an additional 4 or 8 d prior to filtration of the supernatants, lysis of the THP-1 cells, and culture of the liberated *M. tuberculosis*.

**Determination of CFUs**

For the macrophage lysates, a series of 6-fold dilutions were prepared by combining 50 μl cell lysate (500 μl 0.25% SDS plus 500 μl 7H9 medium) with 450 μl 7H9 medium dilution broth after 1 h, 4, or 8 d of infection.

**TUNEL assay**

Cell suspensions (0.5 ml) were grown on four-well slides, followed by differentiation with PMA and infection with *M. tuberculosis* as described previously (59). For each condition, triplicate slides were prepared. The in situ cell death detection kit was used to assay for apoptosis at the single-cell level based on labeling of DNA strand breaks and followed according to the manufacturer’s instructions. Briefly, postinfection with *M. tuberculosis* in glass-chambered slides, the medium was removed, the cells were washed with 50% medium and 50% 1X PBS, and fixed in 4% paraformaldehyde solution (pH 7.4) for 1 h at room temperature, followed by incubation with a blocking solution containing 3% H2O2 in methanol for 10 min at room temperature. The slides were rinsed with 1X PBS and incubated with a permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate solution) for 10 min. They were then rinsed twice with 1X PBS, and 50 μl TUNEL reaction mixture was added to each sample and allowed to incubate for 1 h in a humidified chamber located in a 37°C incubator. The slides were rinsed three times with 1X PBS, and 50 μl Converter-POD substrate (Roche Diagnostic Systems, Indianapolis, IN) was added to each sample and incubated for 30 min in a dark, humidified atmosphere at room temperature. After the slides were washed with 1X PBS three times, 100 μl diaminobenzidine substrate was added and incubated for 2–5 min at room temperature until a brown color developed and then visualized by a light microscope. The slides were rinsed with 1X PBS and dehydrated, and a cover slip was mounted using Permount (Fisher Scientific, Pittsburgh, PA). Enumeration of apoptotic cells was performed by light microscopy, counting ~5000 cells per condition per experiment. The positive control for apoptosis was prepared from fixed and permeabilized cells that were then treated with a DNase solution to induce strand breakage for 10 min. The negative control was incubated with label solution (a nucleotide mixture in reaction buffer) for 30 min.

**Activated caspase-3 assay**

During the terminal stages of the apoptotic pathway, caspase-3zymogen is cleaved by other caspases into three components: a proregion, a large subunit, and a small subunit. Activated caspase-3 was measured by a commercially available kit that uses a biotinylated caspase inhibitor (biontin-ZV Knightone) that covalently modifies only the large subunit but not the inactive caspase-3 zymogen. With a caspase-3 mAb coated on the microtiter plate to capture total caspase-3 and the biotinylated caspase inhibitor that binds to the large subunit added sequentially following by HRP-streptavidin, only activated caspase-3 is detected.

**Cytokine measurements**

Electrochemiluminescence assays for IL-32, TNF-α, IL-1β, and IL-8 were performed as described previously (43). Human TGF-β and IL-10 levels were measured by ELISA kits (R&D Systems) according to the manufacturer’s instructions.

**Statistical analysis**

Replicate experiments were independent, and where appropriate, summary results are presented as mean ± SD. Differences were considered significant for *p* < 0.05. Group means were compared by repeated-measures ANOVA using Fisher’s least significance difference test.

**Results**

**Stable expression of siRNA–IL-32 sequences in THP-1 monocytes**

Each of the three DNA sequences shown in Table I was cloned into the pSUPER.puro RNAi vector and transfected into THP-1 cells. The transfected cells were selected by culture in the presence of puromycin. Through the actions of RNA polymerase III (60), shRNAs are synthesized, comprised two identical 19-nt sequence motifs in an inverted orientation (forming the stems of the hairpin), separated by a 9-nt loop of nonhomologous sequences (the loop of the hairpin). Double-stranded shRNA then undergo enzymatic cleavage by DICER to ultimately yield single-stranded siRNA fragments that are complementary and bind to the target IL-32 mRNA.

To determine whether this DNA vector-based approach can be successfully transferred to human monocyte THP-1 cells, we first transfected the pmaxGFP vector by electroporation. By fluorescent microscopy, transfection efficiency was determined to be ~30% (data not shown). Each of the three DNA fragments encoding shRNA–IL-32, referred to in this study as shRNA–IL-32 (clone 5), shRNA–IL-32 (clone 6), and shRNA–IL-32 (clone 7)—was transfected separately into wild-type (WT) THP-1 cells. The transfected cells were cultured and selected in the presence of puromycin as described in Materials and Methods. Confirmation of knockdown for IL-32 expression

Inhibition of endogenous IL-32 production in the puromycin–selected, siRNA–IL-32–containing cells was confirmed by three methods. First, RT-PCR for IL-32 transcripts was performed after stimulating THP-1 cells expressing one of the three shRNA–IL-32 sequences or

<table>
<thead>
<tr>
<th>Construct</th>
<th>5’-Antisense Sequence/Hairpin Loop/Sense Sequence/Termination-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>psiRNA-IL-32-5</td>
<td>5’-agtctAGAGATTGGCCCGGTATCTaactcagatTTAATTaatAGCGGACTGTCTTaaaA-3’</td>
</tr>
<tr>
<td>psiRNA-IL-32-6</td>
<td>5’-agtctAGAGATTGGCCCGGTATCTaactcagatTTAATTaatAGCGGACTGTCTTaaaA-3’</td>
</tr>
<tr>
<td>psiRNA-IL-32-7</td>
<td>5’-agtctAGAGATTGGCCCGGTATCTaactcagatTTAATTaatAGCGGACTGTCTTaaaA-3’</td>
</tr>
<tr>
<td>pScramble-siRNA</td>
<td>5’-agtctAGAGATTGGCCCGGTATCTaactcagatTTAATTaatAGCGGACTGTCTTaaaA-3’</td>
</tr>
</tbody>
</table>

Shown are three dsDNA constructs that encode three distinct shRNA–IL-32 clones or a scrambled shRNA sequence. Each DNA construct was annealed and ligated into the pSUPER.puro shRNA vector with the HindIII and BglII restriction sites underlined. The underlined sequences at the 5’ and 3’ ends are for directional cloning into the pSUPER.puro shRNA vector. See Materials and Methods for additional details.
a scrambled shRNA sequence with 10 U/ml IFN-γ for 18 h. For the PCR reactions, two different sets of sense and antisense primers shown in Materials and Methods were used. As shown in Fig. 1A (lanes 1–4), IL-32 transcripts were not detected in any of the THP-1 clones under basal conditions. In THP-1 cells expressing the scrambled shRNA sequence (shRNA–IL-32 Scr), the IL-32 mRNA level was increased following stimulation with 10 U/ml IFN-γ for 18 h (Fig. 1A, lane 5). As can be seen, two separate RT-PCR products were present, corresponding to the two different primer sets used. By contrast, IFN-γ–stimulated IL-32 mRNA production was significantly inhibited in the three THP-1 cell lines expressing shRNA–IL-32 sequences, with the greatest inhibition in the cell line stably expressing the shRNA–IL-32 (clone 7) (Fig. 1A, lanes 6–8). RT-PCR for GAPDH revealed equal loading of samples (Fig. 1A, bottom panel).

Second, we confirmed these RNA studies by determining IL-32 protein expression by Western blot analyses. WT THP-1 cells and THP-1 cells expressing scrambled shRNA or one of the three shRNA–IL-32 sequences were stimulated with IFN-γ or infected with M. tuberculosis H37Rv. After 24 h, the cells were lysed, and 20 µg nuclear-free whole-cell lysates from each condition was separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and immunoblotted with an Ab directed against IL-32. This Ab recognizes all known isoforms of IL-32 but has the greatest affinity for IL-32α. As shown in Fig. 1B (lane 1, left panel and right panel), there was no detectable IL-32 protein in unstimulated WT THP-1 cells. Stimulation of WT THP-1 cells or THP-1 cells expressing the scrambled shRNA with 10 U/ml IFN-γ for 24 h resulted in a significant increase in the level of IL-32α protein (Fig. 1B, left panel, lanes 2 and 3). However, IFN-γ stimulation of each THP-1 cell line expressing one of the three shRNA–IL-32 sequences showed significant inhibition of IL-32α production (Fig. 1B, left panel, lanes 4–6). As was seen for IL-32 mRNA expression, THP-1 cells expressing shRNA–IL-32 (clone 7) exhibited the greatest reduction in IL-32 protein levels. Infection of WT THP-1 cells or THP-1 cells expressing the scrambled shRNA with M. tuberculosis H37Rv for 24 h resulted in a significant increase in the level of IL-32α (Fig. 1B, right panel, lanes 2 and 3). By contrast, there was significantly less IL-32 in THP-1 cells expressing shRNA–IL-32 sequences following M. tuberculosis infection (Fig. 1B, right panel, lanes 4–6). Immunoblotting for β-actin revealed roughly equal loading of proteins.

Third, after stimulation with IFN-γ, the cells were lysed, and the lysates were assayed for IL-32α by electrochemiluminescence using the same Ab used for the Western blot analyses (43). As shown in Fig. 1C, THP-1 clones expressing one of the three shRNA–IL-32 sequences showed significant inhibition of IFN-γ–induced IL-32α production compared with that of cells that expressed the scrambled shRNA. Little or no IL-32α was detected in the supernatant. We conclude from these studies that stable expression of shRNA–IL-32 is an efficient method to inhibit IL-32 production induced by IFN-γ or M. tuberculosis.

**Knockdown of IL-32 inhibits M. tuberculosis induction of proinflammatory cytokines**

We next determined whether cytokines induced by M. tuberculosis were affected by knockdown of IL-32. WT THP-1 cells or THP-1 cells expressing scrambled shRNA or one of the three clones of shRNA–IL-32 sequences were either uninfected or infected with M. tuberculosis H37Rv for 6 and 24 h. After the indicated period of incubation, cell culture supernatants were sterile-filtered to remove live M. tuberculosis bacteria and assayed for cytokine levels. As shown in Fig. 2B, in the three clones of THP-1 cells that exhibited reduced IL-32 levels, M. tuberculosis induction of TNF-α was significantly decreased by ~35 and 60% after 6 and 24 h of infection, respectively, compared with those of WT THP-1 cells and THP-1 cells expressing the scrambled shRNA. Similarly, production of IL-1β and IL-8 were substantially less (by ~30–60 and ~40–50%, respectively) at 6 and 24 h following infection with M. tuberculosis H37Rv in cells with reduced IL-32 compared with those of WT THP-1 cells and THP-1 cells expressing the scrambled shRNA (Fig. 2B, 2C). There was little or no induction of TGF-β with M. tuberculosis infection by THP-1 cells expressing either scrambled shRNA (open bars) or shRNA–IL-32 (clone 7) (closed bars) compared with unstimulated cells (open bars).

**FIGURE 1.** Inhibition of endogenous IL-32 production in THP-1 cells by siRNA. A, THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clones 5, 6, or 7) were left unstimulated or stimulated with 10 U/ml IFN-γ for 18 h. RT-PCR was performed with two separate primer sets for IL-32 as well as for GAPDH. The data shown are representative of three independent experiments. B, THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clones 5, 6, or 7) were left unstimulated, stimulated with 10 U/ml IFN-γ for 24 h, or infected with M. tuberculosis for 24 h. Whole-cell lysates were prepared and sterile-filtered, and Western blot analyses were performed for IL-32. The blots were also probed for β-actin. The data shown are representative of three independent experiments. C, THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clones 5, 6, or 7) were left unstimulated or stimulated with 10 U/ml IFN-γ for 24 h. The cells were then lysed, and the amount of IL-32α protein in the nuclear-free whole-cell lysates was measured by electrochemiluminescence. The data shown are the mean ± SD of three independent experiments. ***p < 0.001, compared with bar 2.
Knockdown of endogenous IL-32 inhibited *M. tuberculosis* induction of proinflammatory cytokines. WT THP-1 cells or THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clones 5, 6, or 7) were left uninfected or infected with *M. tuberculosis* for 6 or 24 h. The supernatants were collected, sterile-filtered, and assayed for TNF-α, IL-1β, IL-8, TGF-β, and IL-10. A, TNF-α production with *M. tuberculosis* infection. B, IL-1β production with *M. tuberculosis* infection of WT THP-1 cells and THP-1 cells expressing scrambled shRNA (shRNA Scr) or shRNA–IL-32 (clones 5, 6, or 7). The data shown are mean ± SD of three independent experiments. **p < 0.01; ***p < 0.001; compared with the corresponding THP-1 cells expressing the scrambled shRNA. C, IL-8 production with *M. tuberculosis* infection of WT THP-1 cells and THP-1 cells expressing scrambled shRNA or shRNA–IL-32 (clones 5, 6, or 7). D, TGF-β production following *M. tuberculosis* infection or pravastatin stimulation of THP-1 cells expressing scrambled shRNA (open bars) or shRNA–IL-32 (clone 7) (closed bars). E, IL-10 production with *M. tuberculosis* infection of THP-1 cells expressing scrambled shRNA (open bars) or shRNA–IL-32 (clone 7) (closed bars). The data shown are the mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; compared with the corresponding THP-1 cells expressing the scrambled shRNA.

bars) (Fig. 2D). However, these cells were able to produce TGF-β upon stimulation with pravastatin (Fig. 2D) (61). IL-10 production increased with *M. tuberculosis* infection, but there was no significant difference between cells that expressed scrambled shRNA (open bars) and those that expressed shRNA–IL-32 (clone 7) (closed bars) (Fig. 2F). We conclude from these studies that IL-32 enhances the production of proinflammatory cytokines induced by *M. tuberculosis* but has little or no effect on the expression of the anti-inflammatory cytokine IL-10.

**Silencing of endogenous IL-32 increases the recovery of *M. tuberculosis* H37Rv from infected THP-1 cells**

To determine whether a reduced level of endogenous IL-32 impacts the growth of intracellular *M. tuberculosis*, THP-1 cells expressing one of the three shRNA–IL-32 sequences were infected with *M. tuberculosis* H37Rv for 1 h (day 0), 4 d, and 8 d. At specified times, the cells were lysed, and *M. tuberculosis* was cultured and quantified. For easier visualization, Fig. 3 comprises three separate graphs, each showing the number of *M. tuberculosis* cells cultured from control THP-1 cells transfected with the control empty vector (open squares) or expressing the scrambled shRNA sequence (semiclosed diamonds). Enumeration of *M. tuberculosis* bacteria from each of the three THP-1 transfected cell lines containing shRNA–IL-32 is presented in separate graphs (closed circles) and superimposed over the data for both types of control cells. As can be seen, the number of intracellular *M. tuberculosis* bacteria significantly increased after 4 d in each of the three THP-1 clones knocked down for IL-32 compared with that in the control THP-1 cells. At 8 d postinfection, the number of intracellular organisms was significantly greater in THP-1 cells with silenced IL-32 (clones 5 and 7) compared with that in the control cells. We conclude from these studies that endogenous IL-32 decreases the number of intracellular *M. tuberculosis* bacteria.

IL-32α or IL-32γ induces macrophage apoptosis in the presence of *M. tuberculosis* infection

We next addressed a possible mechanism by which IL-32 inhibits the growth of *M. tuberculosis*. Goda et al. (51) showed that IL-32 induces apoptosis in T cells. More recently, IL-32 produced by stromal cells induced apoptosis of cocultured leukemic cells (46). Because apoptosis is an important effector mechanism for killing intracellular *M. tuberculosis* bacteria (62), we measured the effect of exogenous IL-32 on apoptosis of THP-1 cells. In these experiments, THP-1 cells were cultured in medium alone or medium containing 100 ng/ml rIL-32α for 1 h, then infected with *M. tuberculosis* for 24 h, and followed by assay for apoptosis by TUNEL staining. As shown in Fig. 4A, apoptotic cells are stained brown. Compared to unstimulated and uninfected control cells, stimulation of WT THP-1 cells with rIL-32α alone showed a small insignificant increase in apoptosis (Fig. 4B). In cells infected with *M. tuberculosis*, there was a significant increase in apoptosis and a further increase in apoptosis with addition of rIL-32α.

Although the concentration of exogenous IL-32α required to induce apoptosis in infected THP-1 cells was significantly greater than the amount of endogenous IL-32α measured, a direct comparison cannot be accurately made because the samples used to
measure IL-32α concentrations by electrochemiluminescence were cell lysates that reflected intracellular IL-32 levels subsequently diluted by the lysis buffer. Nevertheless, because the IL-32γ isoform is significantly more active than IL-32α in regards to cytokine production (52), we determined whether lower concentrations of rIL-32γ could recapitulate the findings seen with rIL-32α. As shown in Fig. 4C, 50 ng/ml rIL-32γ or M. tuberculosis induced a significant increase in apoptosis. Similar to the data with IL-32α, there was a further increase in macrophage apoptosis when rIL-32γ was added to the cells infected with M. tuberculosis. With an even lower concentration of rIL-32γ (10 ng/ml), there was a trend toward increased apoptosis in the cells stimulated with IL-32, but this did not reach statistical significance (Fig. 4D).

Knockdown of IL-32 abrogates apoptosis induced by M. tuberculosis

To validate the apoptotic response to rIL-32, the level of endogenous IL-32 was reduced by siRNA, and apoptosis in THP-1 cells infected with M. tuberculosis was evaluated. THP-1 cells expressing scrambled shRNA or shRNA–IL-32 (clone 7) were uninfected or infected with M. tuberculosis for 24 h. TUNEL staining was then performed, and the number of apoptotic cells was quantified. As shown in Fig. 5, reduction of the level of endogenous IL-32 partly, but significantly, abrogated the apoptosis induced by M. tuberculosis (compare bar 4 versus bar 2). We conclude from these experiments that endogenous IL-32 contributes to apoptosis in THP-1 cells infected with M. tuberculosis.

Bcl-2 is upregulated and Bax is downregulated by shRNA–IL-32

Bcl-2 and Bax are mitochondrial membrane proteins that promote cell survival or induce apoptosis, respectively. Bax helps to initiate apoptosis by the intrinsic pathway through the release of cytochrome c from the mitochondria into the cytoplasm. Although the extrinsic pathway is considered to be the principal mechanism by which apoptosis serves a host defense function against M. tuberculosis, there is cross-talk between the two canonical apoptotic pathways because caspase-8 of the extrinsic pathway is able to activate upstream mediators of the intrinsic apoptotic pathway. Thus, because IL-32 enhanced apoptosis of infected macrophages, we determined whether reduction in the level of endogenous IL-32 affected expression of Bcl-2, Bax, and cytochrome c. THP-1 cells expressing scrambled shRNA or shRNA–IL-32 (clone 7) were either left uninfected or infected with M. tuberculosis H37Rv for 1–96 h. After the indicated times, the cells were lysed and sterile-filtered, and nuclear-free lysates (20 μg per condition) were separated by SDS-PAGE. After transfer of the proteins onto nitrocellulose membranes, the membranes were immunoblotted with anti–Bcl-2, anti-Bax, or anti-cytochrome c Abs. As shown in Fig. 6A, uninfected THP-1 cells expressing scrambled shRNA had increased expression of Bcl-2 with increasing times of incubation, peaking at ∼72 h of incubation. In THP-1 cells knocked down for IL-32 (shRNA–IL-32, clone 7), there was a similar increase in Bcl-2 expression, although the increase was qualitatively greater than that in the control cells. However, semiquantitative analysis of Bcl-2 expression normalized for β-actin, as shown by bar graphs beneath the immunoblots, revealed that Bcl-2 was only increased in cells silenced for IL-32 at 1 and 48 h postinfection. In both control THP-1 cells and cells with reduced IL-32 levels infected with M. tuberculosis, there was a noticeable decrease in Bcl-2 expression, particularly with longer times of infection, although in the cells with reduced IL-32 levels there was an increase in Bcl-2 expression after 1 h of infection compared with that of the control THP-1 cells.

As shown in Fig. 6B, Bax expression was similar in uninfected THP-1 cells that expressed either scrambled shRNA or shRNA–IL-32 (clone 7). With M. tuberculosis infection, there was a qualitative increase in Bax expression early (i.e., ≤24 h of infection), although there were no significant differences between THP-1 cells that expressed scrambled shRNA and those that expressed shRNA–IL-32 (clone 7).

Cytochrome c expression was also analyzed in cells expressing shRNA–IL-32 (clone 7) and control cells. As shown in Fig. 6C, in uninfected THP-1 cells expressing either scrambled shRNA or shRNA–IL-32 (clone 7), cytochrome c expression was qualitatively similar, although semiquantitative analysis revealed that cytochrome c expression was reduced at later time points of infection in the THP-1 cells silenced for IL-32. In cells infected with M. tuberculosis H37Rv, cytochrome c expression was modestly decreased at the earlier times following infection in cells with reduced IL-32 levels compared with that of the control cells. We conclude from these experiments that reduction of the IL-32 level marginally increased the expression of Bcl-2 and slightly decreased the expression of cytochrome c in THP-1 cells infected with M. tuberculosis.
Caspase-3 activation by M. tuberculosis is enhanced by endogenous IL-32

Caspase-3 is one of the major effector caspases common to both intrinsic and extrinsic pathways of apoptosis. To corroborate the role of IL-32 in inducing apoptosis in macrophages infected with *M. tuberculosis*, we quantified the level of caspase-3 activation in control and IL-32–silenced cells with and without *M. tuberculosis* infection. The level of activated caspase-3 in THP-1 cells infected with *M. tuberculosis* was assessed by ELISA. As shown in Fig. 7, *M. tuberculosis* infection of control THP-1 cells (shRNA Scr) induced caspase-3 activation with increasing time of infection (open circles). By contrast, THP-1 cells with reduced levels of IL-32 (shRNA–IL-32, clone 7) produced significantly less activated caspase-3 at 6 and 24 h following *M. tuberculosis* infection (closed squares). We conclude from these experiments that with *M. tuberculosis* infection of THP-1 cells IL-32, directly or indirectly, is an important activator of caspase-3.

rIL-32γ reduced viability of intracellular *M. tuberculosis*, which was modestly abrogated by a caspase-3 inhibitor

Commercially available rIL-32γ from R&D Systems, rIL-32γ (R&D), in the absence or presence of a caspase-3 inhibitor, was used to further corroborate the IL-32 knockdown studies. THP-1 cells were infected with *M. tuberculosis* H37Rv and simultaneously incubated with 50 ng/ml rIL-32γ (R&D). At 1 h and 2 d post-infection, the cells were washed and lysed, and serially diluted lysates were cultured for *M. tuberculosis* on 7H10 solid agar. As shown in Fig. 8A, rIL-32γ significantly reduced the number of viable *M. tuberculosis* bacteria by nearly 50% (*p* < 0.01). Coincubation with 10 μM caspase-3 inhibitor (z-DEVD-fmk), which fully inhibited caspase-3 activation (data not shown), modestly but significantly abrogated the inhibition in growth (Fig. 8A, *p* < 0.05). To determine whether rIL-32γ (R&D) induced apoptosis of the infected cells, TUNEL staining was performed, and apoptosis was quantified. As shown in Fig. 8B, rIL-32γ (R&D) and *M. tuberculosis* individually and additively induced apoptosis. In the presence of 10 or 20 μM caspase-3 inhibitor (z-DEVD-fmk), there was a significant decrease in apoptosis induced with *M. tuberculosis* plus rIL-32γ (R&D) (*p* < 0.01), although the level of apoptosis did not return to baseline. We conclude from these studies that exogenous rIL-32γ (R&D) can inhibit the growth of intracellular *M. tuberculosis* through induction of caspase-dependent and possibly caspase-independent apoptotic pathways.

**Discussion**

IL-32 is increasingly recognized to have pleiotropic functions. As originally reported by Kim et al. (43), IL-32 can activate inflammatory signaling pathways and induce cytokine production. Consistent with its proinflammatory effects, IL-32 has been implicated in the pathogenesis of autoimmune disorders such as rheumatoid arthritis and Crohn’s disease (63–65). Whereas IL-32 is not seen in the joints of osteoarthritic patients, it is expressed in the inflamed synovia of patients with rheumatoid arthritis (65). In Crohn’s disease, IL-32 contributes to inflammation by synergizing with bacteria-derived ligands (e.g., muropeptides of peptidoglycan) that bind cytosolic sensors (nucleotide-binding oligomerization domains 1 and 2) to augment the production of IL-1β and IL-6 (64). More recently, the IL-32 level was reported to be increased in the lung tissues of patients with chronic obstructive pulmonary disease (COPD), where it localized with TNF-α and correlated with the degree of airflow obstruction (66).

In contrast to its pathogenic role in the aforementioned diseases, IL-32 is an antagonist for HIV (67, 68) and influenza (69, 70).
Transient transfection of HIV-infected PBMCs or U1 cells with siRNA that targeted IL-32 mRNA increased p24 production, indicating that IL-32 may be a natural inhibitor of HIV (68). In the A549 human type 2 epithelial cell line, influenza induced IL-32 production (70). Interestingly, a selective cyclooxygenase 2 inhibitor (NS398) or aspirin inhibited influenza-induced IL-32 production, suggesting that the PGs may play an intermediary role in the induction of IL-32. We showed previously that IL-32 is also induced by \textit{M. tuberculosis} infection of various primary human cells, including PBMCs, monocyte-derived macrophages, monocyte-derived dendritic cells, and blood lymphocytes (56).

In seeking the biological significance of IL-32 in TB, we found that stable expression of siRNA fragments to silence IL-32 protein production in THP-1 cells decreased production of the proinflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\), and IL-8 induced by \textit{M. tuberculosis}, indicating that IL-32 is involved in a complex network of cytokine production with \textit{M. tuberculosis} infection. We recently showed that silencing of IL-32 also reduced LPS or PMA induction of proinflammatory cytokines (71). Furthermore, in THP-1 cells with IL-32 levels reduced by siRNA, there was an increased number of \textit{M. tuberculosis} bacteria recovered. This significant finding implicates IL-32 as a host protective cytokine in the in vitro infection model. Interestingly, the THP-1 clone that had the greatest inhibition of IL-32 protein expression was also associated with the greatest recovery of \textit{M. tuberculosis}.

We demonstrated that a plausible mechanism by which IL-32 inhibited recovery of \textit{M. tuberculosis} is via induction of apoptosis in the infected THP-1 cells. Although the concentration of rIL-32a required to induce apoptosis in THP-1 cells infected with \textit{M. tuberculosis} was more than that produced endogenously, this is likely due to the fact that the measured IL-32 concentration is an underestimation of the intracellular levels due to dilution by the cell lysis buffer. We therefore corroborated these apoptotic studies by showing that a lower concentration of rIL-32\(\gamma\) had a similar

FIGURE 6. Intrinsic pro- and antiapoptotic gene expression in THP-1 cells knocked down for IL-32 and infected with \textit{M. tuberculosis}. \textbf{A}, THP-1 cells expressing the scrambled shRNA (shRNA Scr) or shRNA–IL-32 (clone 7) were left unstimulated or infected with \textit{M. tuberculosis} for variable lengths of time. After the indicated times of infection, the supernatant was removed, the cells were lysed, and the whole-cell lysates were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and immunoblotted for Bcl-2. \textbf{B}, THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clone 7) were similarly treated as in \textbf{A}, and the cell lysates were immunoblotted for Bax expression. \textbf{C}, THP-1 cells expressing the scrambled shRNA or shRNA–IL-32 (clone 7) were similarly treated as in \textbf{A}, and the cell lysates were immunoblotted for cytochrome \(c\) expression. The membranes were also immunoblotted for \(\beta\)-actin. The bar graphs below each set of immunoblots are mean relative density measurements for the Bcl-2, Bax, and cytochrome \(c\) bands normalized for the densities of their corresponding \(\beta\)-actin band. The data shown are representative of three independent experiments. \(\ast p < 0.05\); \(\ast \ast p < 0.01\); \(\ast \ast \ast p < 0.001\); compared with the corresponding condition with THP-1 cells transfected with scrambled shRNA.

FIGURE 7. \textit{M. tuberculosis} induction of activated caspase-3 is inhibited in cells knocked down for IL-32. THP-1 cells expressing the scrambled shRNA (shRNA Scr) or shRNA–IL-32 (clone 7) were infected with \textit{M. tuberculosis} at the indicated times, the cells were lysed, and activated caspase-3 was quantified by ELISA. Data shown are the mean \(\pm\) SD of three independent experiments. \(\ast \ast p < 0.01\) between scrambled shRNA and shRNA–IL-32 (clone 7).
followed by TUNEL assay. Data shown are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

FIGURE 8. rIL-32γ (R&D) reduces intracellular M. tuberculosis and induces apoptosis. A, THP-1 cells were simultaneously infected with M. tuberculosis H37Rv and stimulated with rIL-32γ. Intracellular M. tuberculosis was enumerated at 1 h and 2 d following infection. B, THP-1 cells were incubated with 50 ng/ml rIL-32γ, M. tuberculosis H37Rv, or both with and without 10 or 20 μM caspase-3 inhibitor (z-DEVD-fmk) for 24 h, followed by TUNEL assay. Data shown are mean ± SD of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

apoptotic effect as the higher concentration of IL-32α and that THP-1 cells knocked down for IL-32 had partial but significant abrogation of apoptosis induced by M. tuberculosis. To further confirm these findings and to delineate the role of apoptosis as a host defense mechanism of IL-32, we used commercially available rIL-32γ (R&D) with or without the presence of a caspase-3 inhibitor in our THP-1 infection model. Although rIL-32γ significantly reduced intracellular viability of M. tuberculosis and induced apoptosis and caspase-3 inhibitor significantly abrogated both effects, the degree of abrogation was modest. These findings suggest that the host defense functions of IL-32 are complex and likely involve both caspase-dependent and caspase-independent apoptotic pathways as well as possibly nonapoptotic mechanisms. IL-32 has also been shown to induce apoptosis in other experimental conditions. For example, Marcondes et al. (46) showed that KG1a leukemic cells underwent apoptosis when cocultured with HS5 stromal cells and that apoptosis was due to IL-32 produced by the stromal cells. The abundant expression of IL-32 in lungs of COPD patients (66) may indirectly indicate increased apoptosis because apoptosis is considered to be an important mechanism in the pathogenesis of COPD. However, apoptotic effects of IL-32 are not universally seen because IL-32 is constitutively expressed in various cancer cell lines or cancer tissues, suggesting that IL-32 may not only be antiapoptotic but may also be involved in the pathogenesis of some tumors (48, 72, 73).

Apoptosis is becoming well-established as an important killing mechanism for intracellular M. tuberculosis, although the precise means by which it occurs in killing mycobacteria is complex (2). The extrinsic apoptotic pathway is activated by avirulent M. tuberculosis H37Ra to a greater degree than by the more virulent M. tuberculosis H37Rv strain, accounting in part for the lower virulence in the former strain (74). TNF-α is considered to be an essential mediator of this classical apoptotic pathway in killing of M. tuberculosis and other mycobacteria (2, 6). However, other apoptotic mechanisms and nonapoptotic cell death that have been shown to affect killing of M. tuberculosis or bacillus Calmette-Guérin, including that induced by the interaction of FasL expressed on cytotoxic T lymphocytes with Fas on macrophages infected with M. tuberculosis (75), NO (7), or ATP ligation to the P2X7R receptor (76). Apoptosis is also considered to have both direct and indirect antimicrobial activity against M. tuberculosis. Although direct antimicrobial activity was demonstrated against various mycobacterial strains (74, 77), the mechanism has not been elucidated. One killing mechanism of apoptosis occurs when apoptotic cells containing M. tuberculosis are ingested by uninfected macrophages, resulting in a greater killing ability because this two-step phagocytic process allows the host cells to override the ability of M. tuberculosis to inhibit phagosome acidification or phagosome–lysosome fusion. However, macrophage necrosis, seen when the number of infecting organisms overwhelms the number of phagocytes or when there are insufficient phagocytes to ingest the infected apoptotic cells, is considered to be detrimental to the host through suboptimal intracellular killing and permissive spread of organisms from cell to cell (2, 78, 79).

Although the extrinsic pathway of apoptosis is considered to be an important mechanism by which intracellular M. tuberculosis bacteria are killed (2, 6), our findings of a marginal increase in Bcl-2 and a modest decrease in cytochrome c in THP-1 cells infected with M. tuberculosis knocked down for IL-32 suggest that the intrinsic pathway may play a minor role. Although inhibition of caspase-3 significantly abrogated the inhibitory effects of rIL-32γ on the growth of M. tuberculosis and on apoptosis, the reversal was modest. Because caspase-3 is a common pathway for both intrinsic and extrinsic pathways of classical apoptosis, it remains to be seen whether nonapoptotic mechanisms and/or caspase-independent apoptotic pathways, such as the less well characterized autophagic mechanism of cell death, caspase-independent apoptosis involving apoptosis-inducing factor and endonuclease G, or those involving sequential apoptosis–necrosis through the lysosomal release of proteases such as cathepsin B, cathepsin D, cathepsin L, and/or the calpains (79–81), may also be mechanistically involved in the antituberculous effects of IL-32. However, necrotic cell death of phagocytes does not kill intracellular M. tuberculosis and may in fact serve to propagate the mycobacteria (82–84). It is also likely that the host defense role of apoptosis may be more substantial in vivo because effecrocytosis of apoptotic bodies and subsequent facilitation of Ag presentation can accelerate activation of the adaptive immune response.

The known biological effects of IL-32 suggest that it may also antagonize M. tuberculosis indirectly by other means in vivo. For example, we found that with M. tuberculosis infection knockdown of IL-32 also inhibited M. tuberculosis production of TNF-α, IL-1β, and IL-8, cytokines shown to be important in the host defense against TB (17, 25, 85). Thus, in the in vivo setting, in the presence of numerous other cell types and a network of both immunostimulatory and immunosuppressive cytokines, the role that IL-32 plays in TB infection is likely to be significantly more complex.

In summary, using an in vitro model of infection of human macrophages that were knocked down for IL-32 expression, we showed that IL-32 is an important host defense molecule against M. tuberculosis. Although induction of caspase-dependent apoptosis is likely an important mechanism for the anti-TB effect of IL-32, caspase-independent forms of cell death and nonapoptotic mechanisms may also be involved.
Acknowledgments
We thank Dr. Robert Winn at University of Colorado Denver School of Medicine for helpful discussions. We are grateful to Dr. Mark Geraci at University of Colorado Denver School of Medicine, Dr. Greg Downey at National Jewish Health, and Dr. Edward Dempsey at the Denver Veterans Affairs Medical Center for their unwavering support for this project.

Disclosures
The authors have no financial conflicts of interest.

References