Supporting Information

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SI Materials and Methods

Generation of SPC-IL-32γTg Mice and Confirmation Methods. Using the SPC promoter in a Tg model is a robust and widely used method to express human genes within the mouse lung (1). To construct the SPC-IL-32γ plasmid, the full-length IL-32γ cDNA was excised from previously described plasmid pTARGET/IL-32γ (2) by digestion with EcoRI and subcloned into the SPC expression vector pUC18SPC3.7, a gift from Stephan W. Glasser (University of Cincinnati, Cincinnati). The resultant plasmid pSPC-IL-32γ, a 3.7-kb DNA consisted of the *SPC* promoter, *IL-32γ* gene, a small t intron from SV40 and the SV40 polyadenylation sequence (Fig. S1*A*).

SPC-IL-32γTg founders were generated at the Mouse Genetics Core Facility at NJH. In brief, the pSPC-IL-32γ plasmid was digested by NdeI and NotI restriction enzymes. The resulting SPC-IL-32γ DNA fragment was purified and microinjected into pronuclei of zygotes isolated from C57BL/6 mice. The surviving zygotes were transferred into the oviducts of pseudopregnant ICR foster mothers. Resulting offspring were screened by tail DNA analysis for the presence of the *SPC-IL-32γ* transgene. Two male founders positive for the *SPC-IL-32γ* transgene were bred with female C57BL/6 WT mice. Successive inbreeding was performed for five generations to select for a litter homozygous for the *SPC-IL-32γ* transgene.

The *SPC-IL-32γ* transgene was determined by both PCR (R&D Systems) and Southern blot. Approximately 2–3 mm of tail from each mouse was harvested and digested overnight at 55 °C using 375 μL lysing buffer [1 M Tris·HCl, pH 7.6 containing 10% (wt/vol) SDS, 5 M NaCl, and 0.5 M EDTA] and 10 μL of 20 mg/mL proteinase K. DNA extraction was performed using standard methods as described previously (3). Extracted DNA was resuspended in sterile water and stored at −20 °C until further use.

For PCR analysis, 100 pg genomic DNA was used as a template in a 30-μL PCR amplification reaction per kit instructions. Amplification was performed by initial denaturing at 94 °C for 4 min, subsequent 20 or 25 cycles of denaturing at 94 °C for 30 s, annealing at 55 °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 separated by 1.5% agarose gel electrophoresis. The following sets of primers were used to detect *SPC-IL-32γ* transgene (Fig. S1*A*): (*i*) TgSpcF: 5′-AGGAACAAACAGGCTTCAAAGCCAAG-3′; (*ii*) TgIL-32R: 5′-GATCTGTTGCCTCGGCACCGTAATC-3′; (*iii*) TgIL-32F: 5′-GATTACGGTGCCGAGGCAACAGATC-3′; and (*iv*) TgSV40R: 5′-GTTTGTCCAATTATGTCACACCACA-3′ (Midland Certified Reagent Co.).

For Southern blot analysis, 10 μg of DNA was digested by HindIII overnight. The DNA was then separated on a 0.7% agarose gel and transferred onto nitrocellulose membranes as described previously (3). The membranes were then hybridized with IL-32γ cDNA labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) (Perkin-Elmer Life Sciences) at 68 °C for 16 h. After washing, the blots were exposed to X-ray films at −70 °C for 2–5 d.

Gene expression for IL-32γ was determined by RT-PCR. Total RNA was isolated from RAW 264.7 cells using the Promega RNA Isolation kit (Promega Biosciences) and from lung homogenates of WT and SPC-IL-32γTg mice at 7–9 wk of age as described (3). The following set of primers was used to detect IL-32γ cDNA: IL-32 (314 bp) sense: 5′-TGA GGA GCA GCA CCC AGA GC-3′ and antisense: 5′-CCGTAGGACTGGAAAGAGGA-3′. The primers used to detect IL-32β cDNA are sense: 5′-AAT CAG GAC GTG GAC AGG TGA TGT-3′ and antisense: 5′-GTG CCA CCA GGT CTG CAG CCG-3′. RT-PCR (Advantage RT-PCR kit, Bio-Rad) was performed for IL-32γ and IL-32β according to the manufacturer's instructions and using the aforementioned set of primers.

Quantitation of IL-32γ in the Lungs. WT and SPC-IL-32γTg mice were killed with pentobarbital and the lungs were removed and homogenized with 1× Cell Lysis Buffer (Cell Signaling Technology), followed by addition of 0.1% Triton X-100. The homogenates were frozen at −80 °C overnight, centrifuged, and the supernatant was analyzed for IL-32γ by ELISA (YbdY Biotech) and normalized for protein content.

Bronchoalveolar Lavage to Determine Lung Cell Differential and to Isolate AMs. Following tracheal cannulation, bronchoalveolar lavage (BAL) of WT and SPC-IL-32γTg mice was performed by instilling 1 mL of sterile 1× PBS into the lungs of each mouse followed by gentle aspiration of the syringe plunger. An average of 0.75 mL of the lavaged saline was recovered from the lungs. Cells from BAL fluid were resuspended in RPMI and counted with a hemocytometer. The cells were stained with Wright-Giemsa stain to identify macrophages, eosinophils, neutrophils, and lymphocytes by light microscopy morphology. After counting at least 500 cells from each lavage under 400× magnification, the percentage and absolute numbers of each cell type was determined.

Alveolar macrophages (AMs) were isolated by BAL from WT and SPC-IL-32γTg mice to perform ex vivo infection with *MTB*. AMs were seeded at 3×10^4 cells per milliliter per well of a 24-well tissue culture plate and incubated overnight with 100 units/mL penicillin G, followed by thorough washing to remove any traces of antibiotic before infection with *MTB*.

Colocalization of *MTB* and Lysosomes. AMs were grown on glass chamber slides for 1 d and then infected with GFP-labeled *MTB*-H37Rv at a MOI of 10 with and without 10 units/mL IFNγ (R&D Systems) for 6–8 h (4). Two hours before the infection time was complete, LysoTracker Red DND-99 (50 nM) was added to the cells to label the lysosomes (Invitrogen/Molecular Probes) (5). After infection time was complete, the medium was removed, the cells were fixed with 4% paraformaldehyde, washed with 1× PBS, stained and mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen), and placed in the dark at 4 °C until use. The cells were then viewed at 400× under oil immersion with an inverted Zeiss 200 M microscope with a 175 W xenon lamp in a DG4 Sutter instruments lamp housing (Carl Zeiss). The Cy3 filter was used to detect the lysosomal staining and the fluorescein (FITC) filter was used to detect the GFP-labeled *MTB* (4). Fifteen random pictures were taken per well. For each picture taken, the number of GFP-*MTB* containing cells was counted as well as the number of cells with GFP-*MTB* that colocalized with lysosomes. For each condition, the percentage of cells with colocalization of GFP-*MTB* and lysosomes was then calculated = number of cells with colocalization of GFP-*MTB* and lysosomes divided by the number of GFP-*MTB*–infected cells.

RAW 264.7 Cell Culture, Plasmid Transfection, and Infection with GFP-*MTB*. The 1×10^6 RAW 264.7 cells were transfected with 1 μg of an empty vector pCDNA3-CMV, pCDNA3-CMV-*IL-32γ*, or pCDNA3-CMV-*IL-32γM* using an Amaxa nucleofector kit V (Lonza) according to the manufacturer's instructions and seeded on 2-cm dishes at a density of 2×10^5 cells per well and incubated in a 5% CO_2 incubator at 37 °C. The donor site of *IL-32γM* is mutated from a GU to AU such that there is no splicing of the mRNA of IL-32γ to IL-32β (6). pMAX-GFP provided by Amaxa was used as a positive control to estimate transfection efficiency.

Transfection of *IL-32γ* gene was validated by RT-PCR using primers noted above. RAW 264.7 cells (0.4×10^5 cells per well) were then transfected with the aforementioned plasmid constructs in four-well chamber slides and then infected with GFP-labeled *MTB* H37Rv at a MOI of 10. After 1 h, the cells were washed, cultured for 2 and 4 d at 37 °C in 5% ambient CO_2, and then fixed with 4% paraformaldehyde. The cells were then stained and mounted with ProLong Gold antifade reagent with DAPI. The images were captured using an inverted Zeiss 200 M microscope (Carl Zeiss). The number of the cells with internalized GFP-*MTB* was quantified by fluorescent microscopy, counting at least 500 consecutive macrophages per condition, and calculating the percentages of macrophages that contained GFP-*MTB*.

Aerosol *MTB* Infection. Pathogen-free 8-wk-old WT C57BL/6 female mice were purchased from The Jackson Laboratory. SPC-IL-32γTg mice were initially maintained at the Biological Resource Center at National Jewish Health (NJH); 8-wk-old Tg mice were transferred to the Biosafety Level 3 facility at Colorado State University (CSU) for aerosol infection with *MTB*. All experimental protocols were approved by the animal care and use committees of NJH and CSU, and conform to NIH guidelines.

A total of 25 WT and 25 SPC-IL-32γTg female mice were infected with a low-dose aerosol of *MTB* W-Beijing HN878 using a Glas-Col aerosol generator (Terre Haute, Inc.), calibrated to deliver ~100 bacteria into the lungs of each mouse (7, 8). After 1, 10, 30, and 60 d of infection, five mice in each group were killed to quantify bacterial burden in the lungs and spleens and to perform immunological analysis of the lungs and regional mediastinal lymph nodes (7, 8). The remaining five WT and five SPC-IL-32γTg were followed for survival for up to 200 d after infection.

Histological Analysis. Lung and splenic tissues from infected WT and SPC-IL-32γTg mice were evaluated. Five mice for each of three time points (10, 30, and 60 d postinfection) were examined for a total of 15 infected WT mice and 15 infected SPC-IL-32γTg mice. Lesion areas were quantified relative to total lung (or splenic) area using a stereology-based area fraction fractionator method (under 20× magnification) by a blinded investigator. The left caudal lung lobe and spleen from each mouse were collected at necropsy and fixed in freshly prepared 4% paraformaldehyde in PBS. Randomly selected lung and splenic tissue sections were embedded in paraffin and cut to 5-μm thickness. Tissue sections were mounted on glass slides, deparaffinized, and stained with H&E. A total of 8–12 fields were randomly selected by the computer and a counting frame (2,000 μm^2) containing probe points with a grid spacing of 200 μm was used to define the areas containing inflammatory cells. The data are expressed as the mean ratio of lesion area to lung or splenic area of all of the animals within a treatment group. The relative area of necrosis was also determined for the total area affected by lung inflammation.

Flow Cytometric Analysis of Cell Surface Markers. Single cell suspensions of lungs and mediastinal lymph nodes from each mouse were incubated with monoclonal antibodies labeled with various fluorochromes at 4 °C for 30 min in the dark as previously described (9). Antibodies directed against the following surface markers were used in this study: CD4 (clone GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), NK1.1 (Pk136), Gr-1 (RB6-8C5), DEC205 (NLDC-145), MHCII (I-A/I-E) (M5/114.15.2), and isotype control rat IgG2a, rat IgG2b, rat IgG1, and mouse IgG1. These monoclonal antibodies were purchased from BD Biosciences Pharmingen as direct conjugates to FITC, PE, PerCP, APC, eFluor 450, Alexa-Fluor 700, or Qdot 800. All samples were analyzed on a Becton Dickinson LSR-II instrument, and data were analyzed using FACSDiva v5.0.1 software. Cells were gated on lymphocytes based on characteristic forward and side scatter profiles. All of the analyses were performed with acquisition of a minimum of 200,000 events. The gating techniques were performed as previously described (7, 9, 10).

Intracellular Cytokine Measurements. Cells isolated from the lungs and mediastinal lymph nodes of each mouse were stimulated for 4 h with the combination of anti-CD3 and anti-CD28 in the presence of Golgi Stop (BD). After incubation, cells were harvested and stained for the cell surface markers indicated above, and thereafter the same cell pellets were resuspended in permeabilization buffer (Foxp3 staining buffer set; eBiosciences) and incubated for 30 min at room temperature. Cells were washed again and resuspended in perm/wash buffer containing labeled monoclonal antibodies against Foxp3 (FJK-16s), IL-17A (eBio17B7) and IL-17F (eBio18F10), TNFα (MP6-XT22), IFNγ (XMG1.2), IL-10 (JES5-16E3), Bcl-2 (BCL/10C4), caspase-3 (C92-605), or isotype control and incubated for 30 min on ice. The cells were then washed twice and resuspended in PBS containing 0.05% sodium azide before analysis.

Immunohistochemical Staining and Semiquantitation. Immunohistochemistry (IHC) was performed on *MTB*-infected WT and IL-32γTg mouse lungs ($n = 3$ each). In addition, following Institutional Review Board (IRB) approval, archived surgical lung tissues were retrieved from seven patients with multidrug-resistant TB and from four control individuals who had lung resection for localized conditions other than TB (two carcinoid tumors, one adenocarcinoma, and one metastatic synovial tumor) as previously described (11). All study participants were HIV negative. Peripheral blood mononuclear cells (PBMCs) were also obtained from five healthy volunteers after IRB approval.

In brief, formalin-fixed, paraffin-embedded lung tissues were cut into 5-μm sections, mounted on slides, deparaffinized, and rehydrated. High-temperature epitope retrieval was performed. Primary antibody specific for IL-32 (1:100, YbdY Biotech and LifeSpan BioSciences), CD68 (1:100, Vector Lab), CD3 (1:100, EMD Millipore), or CD20 (1:100, Vector Lab) was incubated with the lung tissues at 4 °C overnight. Nonimmune IgG was used as a negative control. The Vectastain Elite ABC kit (DAKO) was used according to the manufacturer's instructions. Nuclei were counterstained with Mayer's hematoxylin.

For quantitative immunohistochemical expression analysis, individual slides were scanned using the Aperio ScanScope (Aperio Technologies). The digital images were analyzed using the Aperio ImageScope software. For each tissue examined, a region of interest containing positive signals was manually demarcated by a single investigator (X.B.) at 20× magnification. A preprogrammed algorithm for analysis of IHC staining allowed for identification of positive signals. Ten random areas from the demarcated region of interest were selected from each slide. The fraction of positive signal count was performed, averaged for each slide, and the mean values were recorded.

Statistical Analysis. A parametric method, Student's *t* test was used to assess statistical significance between groups. Group means were compared by repeated-measures ANOVA using Fisher's least significant difference test or by two-way ANOVA with Bonferroni's post hoc test.

1. Vuillemenot BR, Rodriguez JF, Hoyle GW (2004) Lymphoid tissue and emphysema in the lungs of transgenic mice inducibly expressing tumor necrosis factor-alpha. *Am J Respir Cell Mol Biol* 30(4):438–448.
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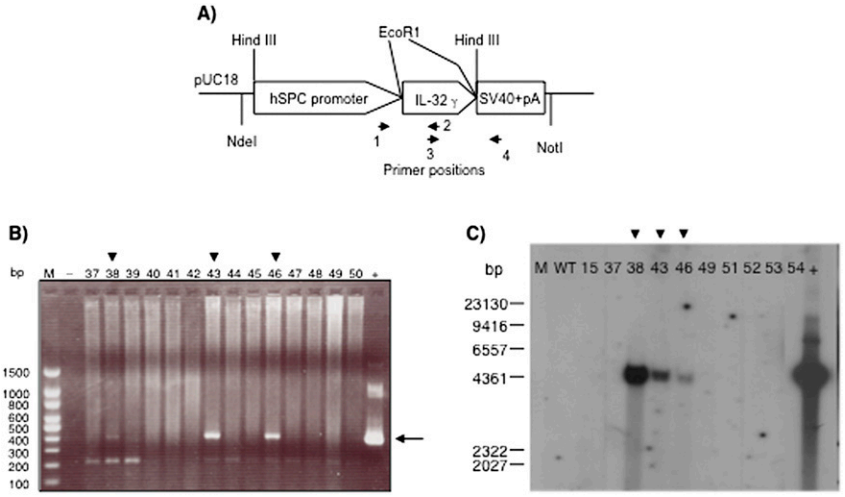

Fig. S1. Integration of human IL-32γ gene into C57BL/6 mice. (*A*) Schematic showing the placement of the human *IL-32γ* gene within the expression fragment containing the human SPC promoter. (*B*) PCR analysis on tail DNA from founder mice using TgSPC-F and TgIL-32R primers. The expected PCR product of 420 bp (arrow) was found in three founder mice (nos. 38, 43, and 46). (–) = PCR of WT mouse tail DNA; (+) = PCR of hSPCIL-32 DNA plasmid. (*C*) Southern blot analysis of the genomic DNA isolated from the tails of first generation Tg mice. Three mice (nos. 38, 43, and 46) carried the expected 4.4-kb band of the *SPC-IL-32γTg* gene. (–) control = tail DNA from wild-type mice, (+) control = hSPC-IL-32 DNA fragment.

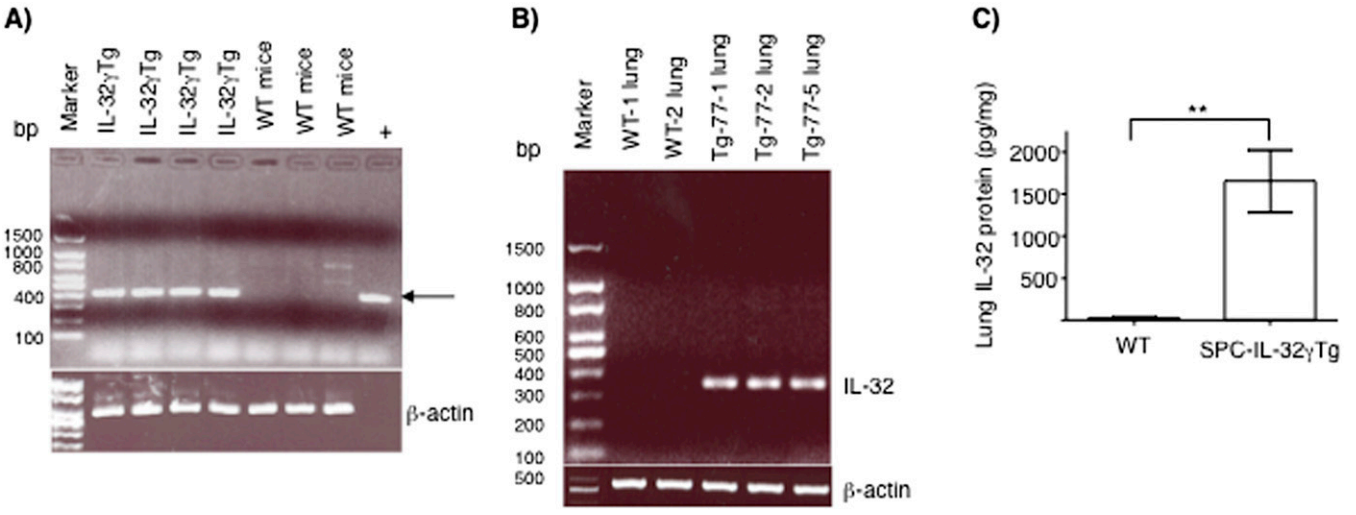

Fig. S2. IL-32 mRNA and protein expression in the SPC-IL-32γTg mouse. (*A*) PCR was performed of tail DNA from four representative SPC-IL-32γTg mice (fifth generation) and three wild-type (WT) C57BL/6 mice using primer set TgSpc-F and TgIL-32R. The arrow indicates the expected PCR product of 420 bp indicating successful integration of the transgene. (+) = hSPC-IL-32γ DNA fragment. (*B*) RT-PCR was performed using RNA isolated from the lungs of two WT and three SPC-IL-32γTg mice. (*C*) IL-32γ levels in the lungs of four WT and four SPC-IL-32γTg mice by ELISA and normalized for total protein (**$P < 0.01$).

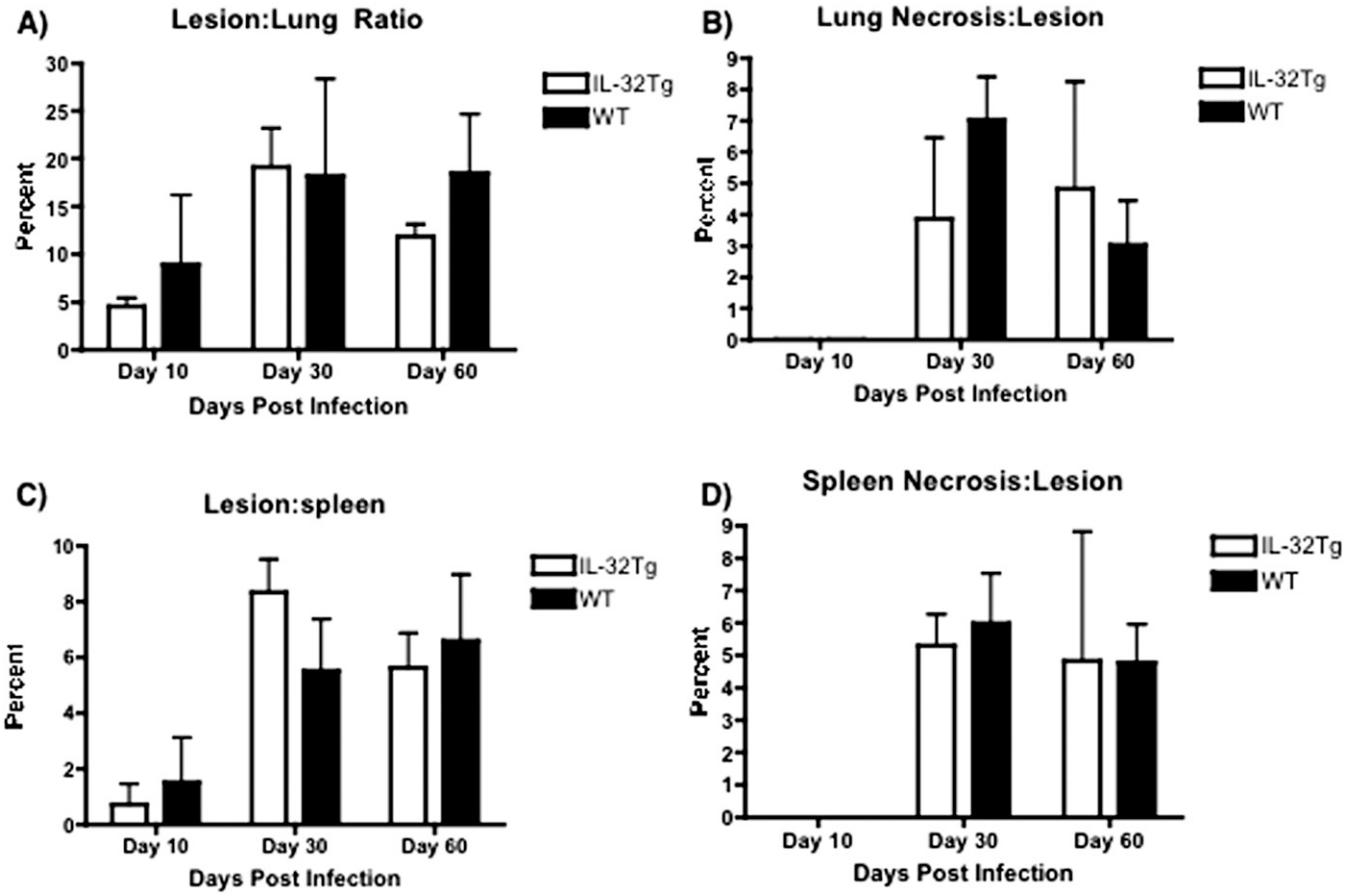

Fig. S3. Lung and spleen lesion scores in the *MTB*-infected WT and SPC-IL-32γ transgenic mice. The lungs and spleens were harvetsted 10, 30, and 60 d after challenge with *MTB* and were stained with H&E. The (*A*) area occupied by TB lesions per total area of the lungs examined and (*B*) area of necrosis per total area of lesions in the lungs were determined by the area fraction fractionator method and reported as percentage. Similarly, in the spleens, the (*C*) area occupied by lesions per total area examined and (*D*) area of necrosis per total area of lesions were determined.

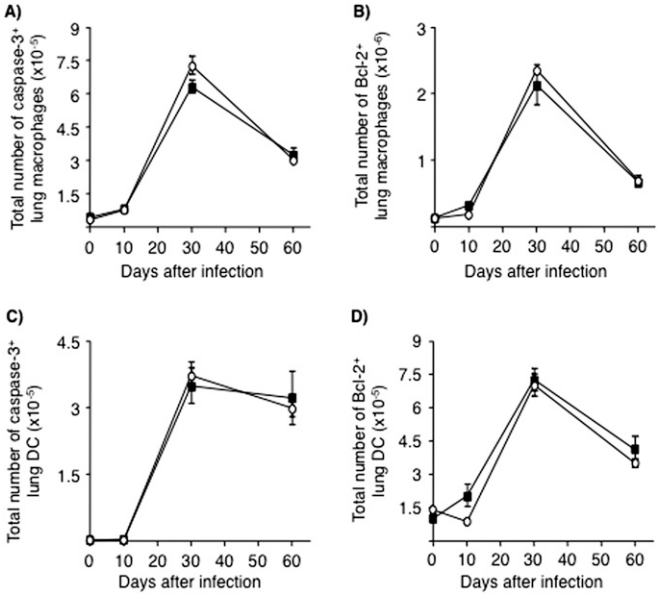

Fig. S4. Analysis of caspase-3$^+$ and Bcl-2$^+$ lung macrophages and dendritic cells of *MTB*-infected WT and SPC-IL-32γ mice. The number of (*A*) caspase-3$^+$ and (*B*) Bcl-2$^+$ lung macrophages of control (open circles) and SPC-IL-32γTg (closed squares) mice are shown over the course of the infection. The number of (*C*) caspase-3$^+$ and (*D*) Bcl-2$^+$ lung dendritic cells of control (open circles) and SPC-IL-32γTg (closed squares) mice are shown over the course of the infection. Results are expressed as the mean number of cells ± SEM with $n = 5$ mice for each time point.

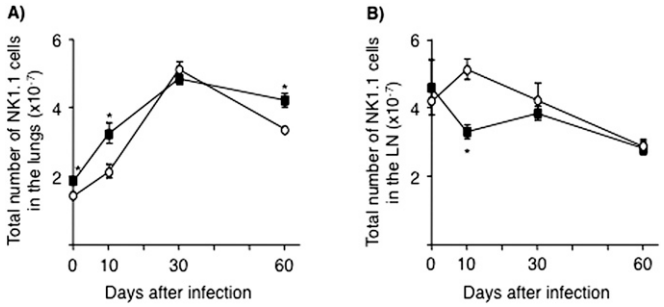

Fig. S5. Analysis of NK cells in the lungs and mediastinal lymph nodes of *MTB*-infected WT and SPC-IL-32γ mice. The number of NK1.1$^+$ cells in the (*A*) lungs and (*B*) mediastinal lymph nodes of control (open circles) and SPC-IL-32γTg (closed squares) mice are shown over the course of the infection. Results are expressed as the mean number of cells ± SEM with $n = 5$ mice for each time point, $^*P < 0.05$.

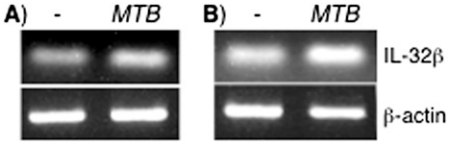

Fig. S6. Expression of IL-32β in the SPC-IL-32γTg mice. SPC-IL-32γTg mice were inoculated with 1.5×10^7 dead *MTB* W-Beijing HN878 in 50 μL 7H9 medium or 50 μL 7H9 medium alone intratracheally for (*A*) 7 d or (*B*) 15 d. The lungs were homogenized, total RNA extracted, and RT-PCR was performed for IL-32β using primers listed in *SI Materials and Methods*.

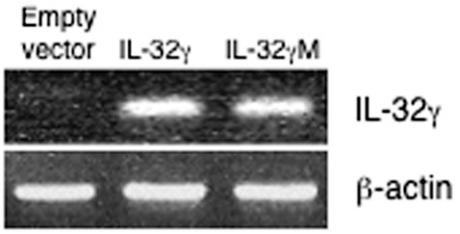

Fig. S7. Detection of IL-32γ mRNA in mouse macrophages transfected with WT *IL-32γ* or *IL-32γ* mutated at the splice site (*IL-32γM*). RT-PCR detection of IL-32γ mRNA expression in RAW 264.7 cells transfected with the empty vector, pCDNA3-CMV-*IL-32γ*, or pCDNA3-CMV-*IL-32γM*.

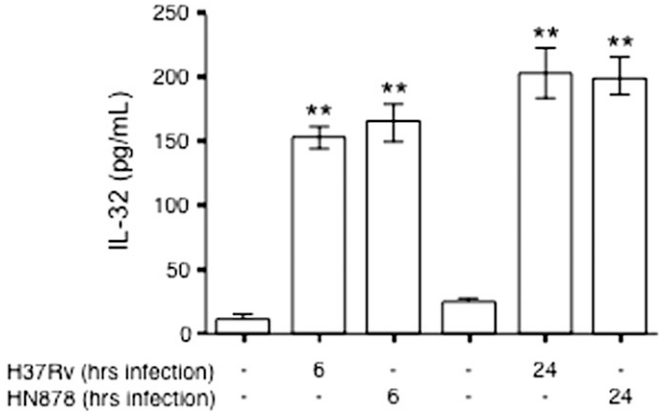

Fig. S8. IL-32 is induced by *MTB* H37Rv and W-Beijing HN878 in human PBMCs. PBMCs isolated from five healthy volunteers were left uninfected or infected with *MTB* H37Rv or W-Beijing HN878. At the indicated time points, the cells were lysed with 1% Triton X-10 and IL-32 measured by ELISA. Data are presented as mean ± SEM ($n = 5$). $^{**}P < 0.01$ compared with uninfected cells.