Supporting Information

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SI Materials and Methods

Reagents. Recombinant human IL-37a, IL-18Rα-Fc, IL-1R8-Fc, TNF-R1-Fc, biotinylated mouse anti-human IL-37 antibody, monoclonal mouse IgG2b, monoclonal mouse anti-human IL-37 antibody, and the Proteome Profiler Human Phospho-Kinase Array kit were from Bio-Techne. HRP-conjugated goat anti-rabbit IgG was from Jackson Immuno Research. HRP-conjugated sheep anti-mouse IgG was from GE Healthcare. The tetramethylbenzidine (TMB) substrate was from eBioscience. For fluorescence-labeled antibodies for flow cytometryic analysis, APC-conjugated anti–IL-1R8 was from Bio-Techne; PE-Cy7–conjugated anti-CD14 and BV-605–conjugated anti-CD3 were from BioLegend; and PE-conjugated anti-CD68 was from eBioscience. FITC-conjugated anti-CD163 was from BD Pharmingen. Recombinant IL-37a isoform (Lys27-Asp192) and anti–phospho-p38 antibody were from Bio-Techne, and anti–phospho-ERK and JNK kinases were from Cell Signaling Technology. Anti–β-actin is from Santa Cruz Biotechnology. Sheep anti-mouse and goat–anti-rabbit HRP-conjugated antibodies were from GE Healthcare. Protease inhibitor mixture tablets were from Roche.

PBMC Culture with Recombinant IL-37 or IL-37 Monoclonal Antibody. PBMCs (0.5×10^6) were cultured per well in 96-well plates in the presence of 100 U/mL penicillin/streptomycin in RPMI with 5% FCS. For the recombinant IL-37 experiment, the cells were pretreated with increasing concentrations of recombinant IL-37 from 100 pg/mL to 100 ng/mL for 2 h before 10 ng/mL LPS was added. For the experiment with IL-37 monoclonal antibody (MoAb), the PBMCs were pretreated with 1 or 0.1 μg/mL IL-37 MoAb for 10 min before 100 ng/mL LPS was added. After 24 h, the supernatants were collected by centrifugation at $1,000 \times g$ for 5 min and were used for direct analysis or stored at −80 °C.

Human M1, M2, and DC Differentiation. As previously described, adherent PBMCs were differentiated with 50 ng/mL human GM-CSF (for M1 cells), 50 ng/mL human M-CSF (for M2 cells), or 50 ng/mL human GM-CSF + 20 ng/mL IL-4 (for DCs) for at least 5 d in the presence of 100 U/mL penicillin/streptomycin in RPMI with 10% FCS (1, 2). After differentiation, the spent medium was removed, and the cells were pretreated with increasing concentrations of recombinant IL-37 from 100 pg/mL to 100 ng/mL for 2 h before 10 ng/mL LPS was added. After 24 h, the supernatants were collected by centrifugation at $1,000 \times g$ for 5 min and were used for direct analysis or stored at −80 °C. Cells were resuspended and lysed in 100 μL 0.5% Triton-X in RPMI and stored at −80 °C for further analysis.

In Vivo LPS Model. Mice were kept in the animal facility for at least 5 d before use. Animals received food and water ad libitum. Mice were treated with a total volume of 200 μL. IL-37b at the doses of 1 μg or the same volume of vehicle control was administered intraperitoneally. Twenty-four hours after the administration of recombinant IL-37, the animals were injected intraperitoneally with 100 μL LPS *E. coli* (055:B5) (Sigma) at 10 mg/kg for 24 h. Using a lower dose of LPS (2.5 mg/kg), mice were pretreated with IL-37 2 h before the LPS dose and euthanized after 4 h for the collection of organs and blood. Cytokines were measured in plasma obtained by gentle centrifugation at $300 \times g$ for 10 min. The organs were homogenized in lysis buffer containing 1% Triton X as described (3), and the supernatant were used for cytokine levels. For experiments with peritoneal lavage, 10 mL PBS was injected in the peritoneal cavity before any organ collection. The lavage was collected after gentle massage of the cavity for efficient diffusion of the fluid. The peritoneal lavage was then centrifuged at $1,000 \times g$ for 5 min, and the supernatants were used for cytokine measurement. For whole-blood cultures, freshly collected whole blood from either vehicle or IL-37–pretreated mice was diluted 1:5 with RPMI medium. Two hundred and fifty microliters of the diluted whole blood was cultured on 96-well round-bottom plates for 24 h before the supernatants were collected for cytokine measurement.

BMDC Culture. Bone marrow cells were obtained from the tibia and femur of either wild-type or IL-1R8–deficient mice. Cells (0.5×10^6) were seeded per well on 96-well plates in RPMI. One hour later, nonadherent cells were washed away and the adherent cells were cultured with 10% (vol/vol) FCS for 5–7 d in the presence of 20 ng/mL mouse GM-CSF and 20 ng/mL IL-4 (4, 5). After differentiation, the spent medium was removed. The cells were pretreated with or without different concentrations of recombinant IL-37 for 2 h before the cells were further stimulated with 20 ng/mL LPS for 24 h. The supernatants were then collected for cytokine measurement. For MAPK phosphorylation assay, about 5×10^6 bone marrow cells were seeded per well on a 24-well plate and differentiated similarly. The cells were pretreated with or without different concentrations of recombinant IL-37 for 2 h before 1 μg/mL LPS was added. Thirty minutes later, the cells were washed once with ice-cold PBS before 1× cell lysis buffer (Cell Signaling) containing protease inhibitor was added. For the mRNA study, the differentiated BMDCs were harvested and further purified for the $CD11c^+$ subset using anti-CD11c microbeads and auto MACS (Miltenyi Biotec). BMDCs were incubated for 24 h with LPS (100 ng/mL) before the mRNA are collected.

Flow Cytometry. For nonpermeabilized cell staining, the cells were stained with anti-SIGIRR (APC), anti-CD14 (PE-Cy7), anti-CD68 (PE), anti-CD3 (BV605), or anti-CD163 (FITC) and the appropriate isotype controls (eBioscience or Bio-Techne) for 30 min at 4 °C. Cells were then washed twice and fixed with 1% formaldehyde. For permeabilized cell staining, the cells were first stained with anti-CD14 (PE-Cy7), anti-CD3 (BV605), or anti-CD163 (FITC) for 30 min at 4 °C and then fixed for 15 min. After washing, the cells were permeabilized using the FIX & PERM Cell Permeabilization Kit (Invitrogen) for 15 min and washed before anti–IL-1R8 (APC) and anti-CD68 (PE) were used for both surface and intracellular staining. Cells were then washed twice further and resuspended in fix buffer for flow analysis.

Cells were analyzed using an LSR-II flow cytometer (BD Immunocytometry Systems). Between 0.1 and 1 million events were collected. Electronic compensation was performed with antibody capture beads (BD Bioscience) stained separately with individual antibodies used in the test samples as described (3). The data were analyzed using the FlowJo Software (Treestar), and biexponential scaling was used in all dot plots. Lymphocytes, macrophages, and monocytes were gated based on their forward and side scatter profile. M1 (CD163−) and M2 (CD163+) cells were selected by the expression of CD163, which was determined earlier as a M2 marker (6–8). Quadrant gates were set using cells stained with isotype controls, and the percentage of SIGIRR+ cells expressing either CD14 or CD68 was determined.

To control for the accuracy and precision of measurements taken during the course of the study, routine quality control was performed on the LSRII using the Cytometer Setup and Tracking (CS&T) feature within BD FACSDiva software version 6.1.2 (BD Biosciences). Voltage, laser delay, and area scaling were

determined using standardized CS&T beads (BD Biosciences), and settings were tracked over time. To verify the laser delay and area scaling determined by CS&T, a manual quality control using rainbow beads was performed daily.

Western Blotting. Human M1 macrophages or mouse BMDCs (5×10^6) were pretreated with or without 0.1 ng/mL IL-37 for 2 h and then stimulated with 1 μg/mL LPS for 30 min before the cell lysates were collected. The total protein concentration was measured using Bradford protein assay kit (Bio-Rad). For Western blot analysis, 25 μg of cell lysates was loaded for each sample and run on SDS/PAGE gel for protein separation. The proteins were transferred to nitrocellulose membranes and then blotted with antibodies for phosphor-p38, -ERK, and -JNK kinases. β-Actin was blotted as the loading control. For human phospho-kinase array study, 100 μg of M1 cell lysates was used for assay following the instructions in the kit (Bio-Techne).

Binding of Recombinant IL-37 to Immobilized IL-1R8-Fc or IL-18Rα-Fc. ELISA plates were coated overnight with 1 μg/mL SIGIRR-Fc or IL-18Rα-Fc. The plate was washed three times and then blocked with 1% BSA in PBS as described before (9). Various concentrations of recombinant IL-37b or IL-37a were added to the wells and incubated for 2 h at room temperature. The wells were washed three times. For IL-37b binding assay, an affinity-purified rabbit anti-human IL-37b serum (3, 10) was added to the wells for 2 h at room temperature. After three washings, the wells are incubated with HRP-conjugated goat anti-rabbit IgG for 2 h. For IL-37a binding assay, wells were incubated with biotinylated mouse anti–IL-37 antibody for 2 h at room temperature and then washed for 20 min with HRP-conjugated sheep anti-mouse IgG. For both IL-37b and IL-37a binding assays, the wells were further washed and incubated with 1× TMB substrate for 20 min before the plates were read at 450 nM for OD values. A currently unrelated TNF-R1 was used as a control.

Steady-State mRNA and Real-Time PCR. For steady-state mRNA study, 5×10^6 human PBMCs or mouse bone marrow cells were seated per well in 24-well plate, and adherent cells were differentiated to M1 or BMDCs as mentioned above. The cells were pretreated with different concentrations of recombinant IL-37 for 2 h before LPS stimulation (1 μg/mL LPS for M1 and 100 ng/mL for BMDCs). After 24 h, the cells were washed one time with PBS and lysed in RLT Plus lysis buffer. Total RNA was collected with the RNeasy mini plus kit from Qiagen. Approximately 300–500 ng of total RNA was reverse-transcribed using SuperScript first-strand synthesis system to obtain the cDNA (Invitrogen). Real-time PCR was then performed on a 7300 real-time PCR system with a cycle of 40 using Power SYBR green PCR Master Mix (Applied Biosystem). PCR using GAPDH-specific primers as an internal control was performed for each RNA sample. The forward primer used for human TNFα real-time PCR is 5′-CCC AGG CAG TCA GAT CAT CTT C-3′ and the reverse primer is 5′-AGC TGC CCC TCA GCT TGA-3′. For human IL-1R8, the forward primer is 5′-TCA GTG GCT CTG AAC TGC AC-3′ and the reverse primer is 5′-GTA CCA GAG CAG CAC GTT GA-3′. For human GAPDH, the forward primer is 5′-TGC ACC ACC AAC TGC TTA GC-3′ and the reverse primer is 5′-GGC ATG GAC TGT GGT CAT GAG-3′. For human IL-18Rα, the forward primer is 5′-CACAGACACCAAAAGCTTCATC-3′ and the reverse primer is 5′-CAC AGT CAC TAG GCA CAC TAC-3′. For mouse GAPDH, the forward primer is 5′-TGC ACC ACC AAC TGC TTA GC-3′ and the reverse primer is 5′-GGC ATG GAC TGT GGT CAT GAG-3′; for mouse IL-1β, the forward primer is 5′-TCA GGC AGG CAG TAT CAC TC-3′ and the reverse primer is 5′-AGG ATG GGC TCT TCA AA-3′. The relative ratio of the mRNA from interested gene to internal control (GAPDH) was calculated as $1/2^{\Delta Ct}$ (interested gene-internal control gene).

Statistical Analysis. Significance of differences was evaluated with Student's two-tail *t* test. LPS-induced cytokine production was set at 100% for each donor unless specified. The mean percentage change for each concentration of recombinant IL-37 was calculated for each donor. The data shown represent the mean percentage change for all of the donors of each condition unless specified.

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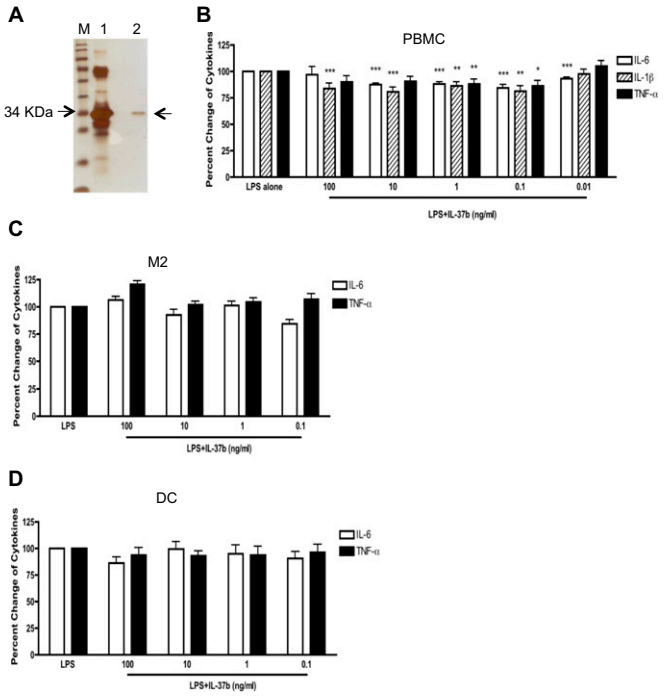

Fig. S1. The effect of recombinant IL-37 on LPS-induced cytokine production in PBMCs, differentiated human M2 macrophages, and DCs. (*A*) Silver staining of SDS/PAGE of recombinant human IL-37b precursor. Lane 1, *E. coli*-derived recombinant IL-37 before HPLC. Lane 2, HPLC purified recombinant IL-37. (*B*) Mean ± SEM of LPS-induced cytokine production in PBMCs with decreasing concentrations of recombinant IL-37b precursor ($n = 13$). (*C*) Mean ± SEM percentage change of LPS-induced cytokine production in M-CSF–differentiated M2 macrophages with IL-37b ($n = 10$). (*D*) Mean ± SEM percentage change of LPS-induced cytokine production in GM-CSF+IL-4–differentiated human DCs ($n = 10$). ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$, compared with LPS alone.

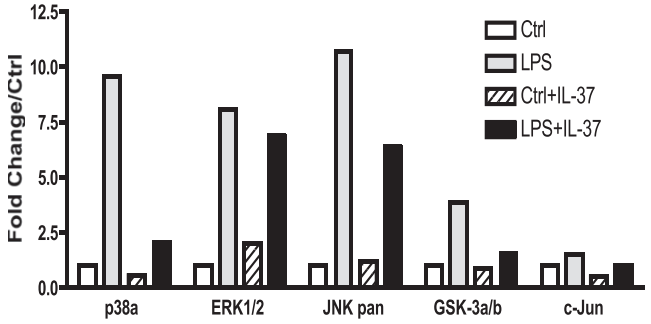

Fig. S2. The effect of IL-37 on LPS-induced selected kinase phosphorylations in M1 cells. M1 macrophages with incubated with 0.1 ng/mL of IL-37 precursor for 2 h, followed by LPS and by lysis buffer for the array. The levels of phosphorylated kinases were detected by Proteome Profiler Human Phospho-Kinase Array kits. The data were analyzed by the quantified pixel intensities representing the individual phosphorylated kinases as shown. One of two independent experiments is shown.

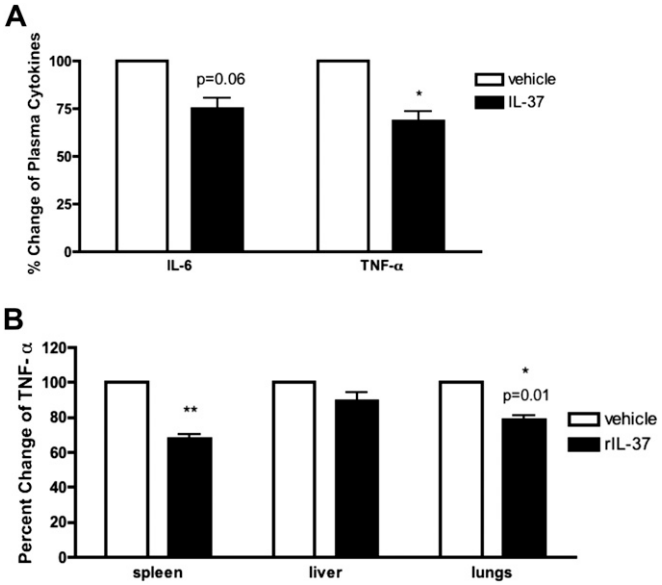

Fig. S3. The effect of IL-37 pretreatment on LPS-induced IL-6 and TNFα. Mice were injected i.p. with 1 μg IL-37 2 h before LPS (2.5 mg/kg). Four hours later, plasma and organs were removed. (*A*) Mean ± SEM plasma levels of IL-6 and TNFα. (*B*) Mean ± SEM. TNFα levels in organ lysates. Data are derived from three separate experiments; $n = 9$ mice total.

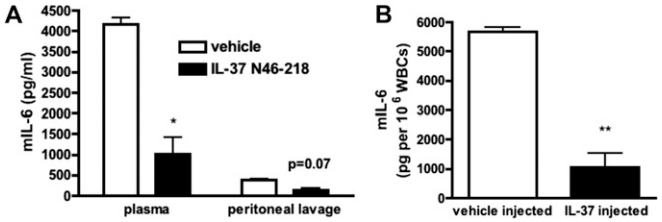

Fig. S4. The effect of recombinant IL-37 N46-218 pretreatment on LPS-induced IL-6. WT mice were injected with 1 μg IL-37N46-218 intraperitoneally (at −24, −16, and −1 hr) before LPS (10 mg/kg). Twenty-four hours later, peritoneal lavage, whole blood, and plasma were collected. (*A*) Mean ± SEM. IL-6 levels of plasma and peritoneal lavage fluid from either vehicle or IL-37–pretreated mice. (*B*) Mean ± SEM. IL-6 levels of whole-blood culture from either vehicle or IL-37–pretreated mice. For both *A* and *B*, $n = 3$.

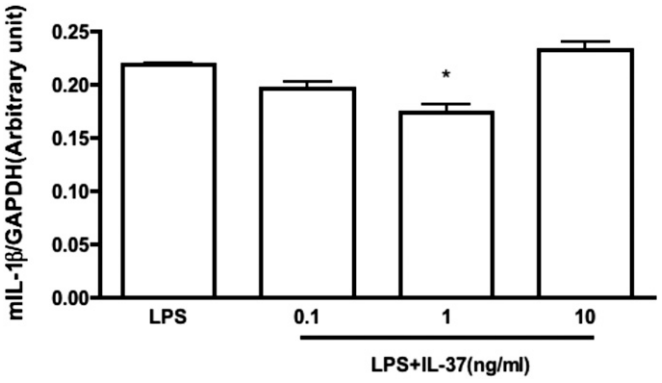

Fig. S5. Dose–response of IL-37 pretreatment on LPS-induced mouse IL-1β mRNA levels in BMDCs. Mean ± SEM. IL-1β steady-state mRNA synthesis induced by LPS (100 ng/mL) with or without recombinant IL-37 ($n = 3$; $^*P < 0.05$).

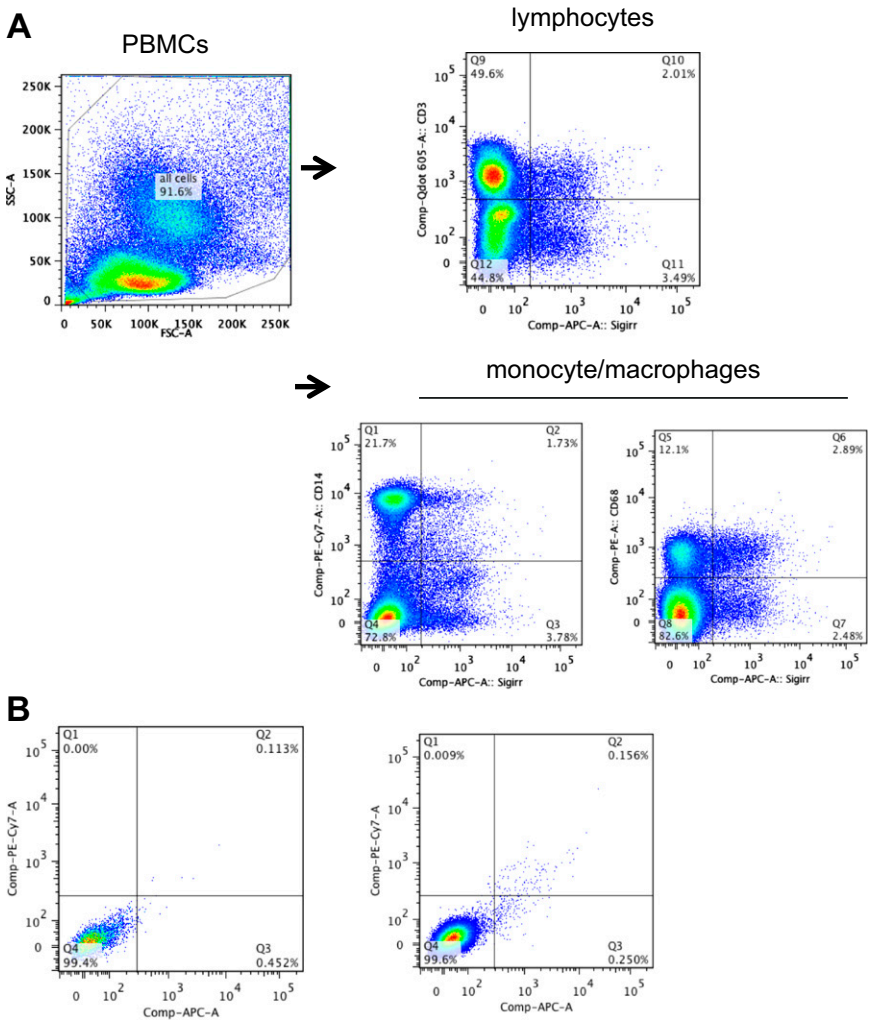

Fig. S6. IL-1R8/SIGIRR expression level in resting or differentiated cell populations. (*A*) Flow cytometric images of the surface expression profiles in lymphocytes (CD3+) and CD14+ or CD68+ monocytes/macrophages in fresh PBMCs. Representative of three independent donors. (*B*) Flow cytometric profiles of the negative controls for IL-1R8/SIGIRR and CD14 expression in nonpermeabilized or permeabilized M1 macrophages.