Supplemental Information for

Interferon-β regulates pro-resolving lipids to promote the resolution of acute airway inflammation

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Materials and Methods

Bacterial DNA. *Escherichia coli* DNA (strain B, Sigma-Aldrich) was purified by extraction with phenol: chloroform: isoamyl alcohol (25:24:1 vol/vol/vol) and ethanol precipitation (1). DNA was resuspended in TE buffer and concentration was measured using Nanodrop at 260 nm. All DNA preparations contained <5ng of LPS per mg DNA by *Limulus* assay.

Neutrophil Isolation and Culture. Venous blood (20 mL, anticoagulated with sodium heparin, 50 U/mL) was obtained from non-smoking apparently healthy volunteers (male and female, 26-65 years) who had denied taking any medication for at least 2 weeks. The Clinical Research Committee at the Maisonneuve-Rosemont Hospital approved the experimental protocols (permit no. 99097) and each blood donor gave written informed consent. Neutrophils were isolated by centrifugation through a Ficoll-Hypaque gradient, sedimentation through dextran (3% wt/vol), and hypotonic lysis of erythrocytes (2). Neutrophils ($5x10^6$ cells/mL, purity>96%, viability>98%, apoptotic<2%) were cultured in RPMI1640 medium supplemented with 10% autologous serum on a rotator with human recombinant IFN-β (12.5 or 50 ng/mL; Peprotech) ± 15-epi-lipoxin A_4 (15-epi-LXA_4, 5S,6R,15R-trihydroxy-7E,9E,11Z,13E-eicosatetaenoic acid, 1 μM, Cayman Chemical) or 17-epi-resolvin D1 (17-epi-RvD1, 7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid, 200 nM; Cayman Chemical), and then challenged with CpG DNA (0.4 or 1.6 μg/mL). In additional experiments, neutrophils were cultured with or without the formyl peptide receptor 1 (FPR1) inhibitor cyclosporin H (1 μM, Tocris) or the ALX/FPR2/ inhibitors *N*-t-Boc-Phe-Leu-Phe-Leu-Phe (Boc-2, 50 μM; MP Biomedicals) or Trp-Arg-Trp-Trp-Trp (WRW4, 5 μM; Tocris) (3) and then challenged with IFN-β and CpG DNA. Concentrations for IFN-β, 15-epi-LXA_4 and 17-epi-RvD1were informed from previous studies (4-7). At the designated time points, neutrophils were processed as described below.

Assessment of Apoptosis. Apoptosis in human neutrophils was assessed with flow cytometry using FITC-conjugated annexin-V (BD Biosciences) in combination with

propidium iodide (Molecular Probes) and the percentage of cells with hypoploid DNA (1, 8). Mitochondrial transmembrane potential was monitored following staining for 15 min with the lipophilic fluorochrome chloromethyl-X-rosamine (CMXRos, 200 nM, Millipore-Sigma) and the fluorescence was analyzed in a FACSCalibur flow cytometer (8).

CD11b and C5aR Expression. Surface expression of CD11b and C5aR (CD88) on human freshly isolated neutrophils or neutrophils cultured with IFN-β (50 ng/mL) and challenged with CpG DNA (1.6μg/mL) for 1 h was assessed using R-phycoerythrin-conjugated mouse anti-human CD11b antibody (clone D12, cat no. 347557, BD Biosciences) and FITC-labeled anti-CD88 antibody (clone: S5/1, cat. no. 344306, BioLegend), respectively, with appropriately labeled isotype-matched (IgG2a) irrelevant monoclonal antibodies. Immunofluorescence was analyzed with a FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences) and was expressed as the percentage of fluorescence of unstimulated cells following corrections with the fluorescence intensity an appropriately labeled, class-matched, irrelevant antibody (7).

Neutrophil Elastase Release. Neutrophil elastase activity in cell-free conditioned media was measured with a colorimetric assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (2mM, Sigma-Aldrich) as a substrate with purified human neutrophil elastase (Athens Research & Technology) as a standard (9).

Phagocytosis and Phagocytosis-Induced Cell Death. For quantitative analysis of phagocytosis, neutrophils were mixed with heat-killed FITC-labeled *E. coli* (K12 strain, cat. no. E2861, Invitrogen) in the presence of 10% autologous serum at a ratio of 7 bacteria per neutrophil for 30 min. The mixture was spun down to remove supernatant and the cells were resuspended in 100 μL ice-cold phosphate-buffered saline containing 0.2% trypan blue to quench extracellular fluorescence (10). Intracellular fluorescence was analyzed with a FACSCalibur flow cytometer and CellQuestPro software.

To assess apoptosis following phagocytosis, neutrophils were cultured for 24 hours with live *E. coli* (7 bacteria per neutrophil, American Type Tissue Culture, ATCC 25922) with or without IFN-β (12.5 or 50 ng/mL). The *E. coli* ratio was chosen to avoid distortion of cell morphology by an overabundance of phagocytosed particles (10). Neutrophils were stained with FITC-conjugated annexin-V or CMXRoS and the fluorescence was analyzed in a FACSCalibur flow cytometer.

Bacterial Killing. Neutrophils were cultured with IFN-β and CpG DNA and then mixed with live *E. coli* at a ratio of 1:1 or 1:7 for 3 h. Bacteria contents in culture media were assessed by growth on tryptic agarose for 24 hours and counting colonies (7).

Western Blot Analysis. Proteins from 10^7 neutrophils were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride (PVDF) membranes, blocked with 5% nonfat milk, and probed with antibodies to Mcl-1 (rabbit polyclonal, cat. no. 16225-1-AP, Proteintech) or actin (C-2, mouse monoclonal, cat. no. sc-8432, Santa Cruz Biotechnology) (8).

Neutrophil 15-epi-LXA$_4$ and RvD1 Secretion. Human neutrophils (10^7 cells/ml) suspended in RPMI 1640 medium supplemented with 2% autologous serum were cultured with IFN-β (50 ng/mL) ± CpG DNA (1.6 μg/mL) for 10 min or 4 h. To study phagocytosis-associated SPM production, neutrophils, suspended in RPMI 1640 medium containing 10% autologous serum (to opsonize bacteria), were cultured with CpG DNA (1.6 μg/ml) and *E. coli* at a ratio of 1:7 for 30 min, centrifuged, resuspended in culture media containing 2% autologous serum and then challenged with IFN-β (50 ng/mL) for 4 h. Conditioned media were collected and analyzed for 15-epi-LXA$_4$ and RvD1 by selective direct ELISAs (Cayman Chemical) following the manufacturer`s instructions.

Murine Acute Lung Inflammation Models. Female C57BL/6 mice (aged 8-14 weeks, Charles River Laboratories, St. Constant, QC, Canada) were housed in pathogen-free conditions. The Animal Care Committee of the Maisonneuve-Rosemont Hospital

approved the experimental protocols (protocol nos. 2015-31 and 2019-1765). All animal experiments were performed in accordance with animal welfare guidelines.

Under isoflurane anesthesia, mice received intratracheal instillation of 5×10^6 colony-forming units (CFUs) *E. coli* (American Type Culture Collection, ATCC 25922) in 50 μL sterile saline with or without simultaneous intraperitoneal injection of CpG DNA (1 μg/g b.w. in 150 μl saline) (7). At 6 h (near the peak of inflammation), mice were treated with carrier-free recombinant mouse IFN-β1 (2.5 ng/g b.w. in 200 μL sterile saline, i.p.; BioLegend, cat. no. 581302), 15-epi-LXA_4 (125 ng/g b.w. in 200 μL saline, i.p., Cayman Chemical) or 17-epi-RvD1 (25 ng/g b.w. in 200 μL saline, i.p., Cayman Chemical) or vehicle (saline, 200 μL), as informed from previous studies (7, 8, 11-13). Some mice were injected cyclosporin H (5 μg/g b.w. in 150 μL saline, i.p.) or WRW4 (1 μg/g b.w. in 150 μL saline, i.p.) (14) 10 min before treatment with IFN-β. Another group of mice was first injected with rat anti-mouse IFN-β antibody (clone 7FD3, cat. no. ab24324, Abcam), rat IgG1 (Abcam) (both at 50ng/g b.w. in 200 μL sterile saline, i.p.) or vehicle, followed by intratracheal instillation of $5x10^6$ live *E. coli.*

Assessment of Inflammation. At the indicated times, mice were killed and the lungs were lavaged (4 times with 1 mL saline containing 5 U/ml sodium heparin) or processed for analysis without lavage. Bronchoalveolar lavage fluid protein and total and differential leukocyte counts were determined using standard techniques (8). Apoptosis in neutrophils (identified as Ly6G-positive cells) was assessed by staining with FITC-conjugated annexin-V (BD Biosciences) using flow cytometry. The percentage of macrophages containing apoptotic bodies was assessed following staining with hematoxylin and eosin (8). In separate groups of mice, the lungs were removed without lavage, and processed for standard histological evaluation or determination of lung dry-to-wet weight ratio and myeloperoxidase, an index of tissue neutrophil infiltration. Myeloperoxidase activity was measured using o-dianisidine as a substrate and human leukocyte myeloperoxidase (cat. no. M6908, Sigma-Aldrich) as a standard (15). Lung bacteria contents were assessed by growth on tryptic agarose using aliquots of lung homogenates (7).

Bronchoalveolar Lavage Fluid Mediators. The first 1 mL cell-free lavage fluid was used to assess selected mediators. Lavage fluid levels of IFN-β were determined by VeriKine-HS Mouse Interferon Beta Serum ELISA Kit (Pestka Biomedical Laboratories, Inc).The assay has no cross-reactivity with mouse IFN-α and IFN-γ. Intra- and inter-assay variations were 5.5 and 8.3%, respectively. 15-epi-LXA_4 and RvD1 were measured by selective direct ELISAs (Cayman Chemical) in accordance with the manufacturer`s protocol. The 15-epi-LXA_4 assay has 26% cross-reactivity with 17-epi-RvD1, >0.4% cross-reactivity with LXA_4 and other pro-resolving lipids. The RvD1 ELISA has 20% and 4% cross-reactivity with 5(S), 6(R)-LXA_4 and 17-epi-RvD1, respectively. Intra- and inter-assay variations were <15% for RvD1 and <12% for 15-epi-LXA_4. BAL fluid cytokine levels were measured using a mouse multiplexed bead-based immunoassay (Bio-Plex Pro^{TM} Coupled magnetic beads, cat. no. 171304070M, Bio-Rad) in accordance with the manufacturer`s protocol as described previously (7).

Statistical Analysis. Results are expressed as mean±SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn`s multiple contrast hypothesis test to identify differences between various treatments. Correlations were assessed by calculating the Spearman rank correlation coefficient. A value of $P<0.05$ was considered statistically significant. Graph Pad Prism 5 was used to calculate statistical differences.

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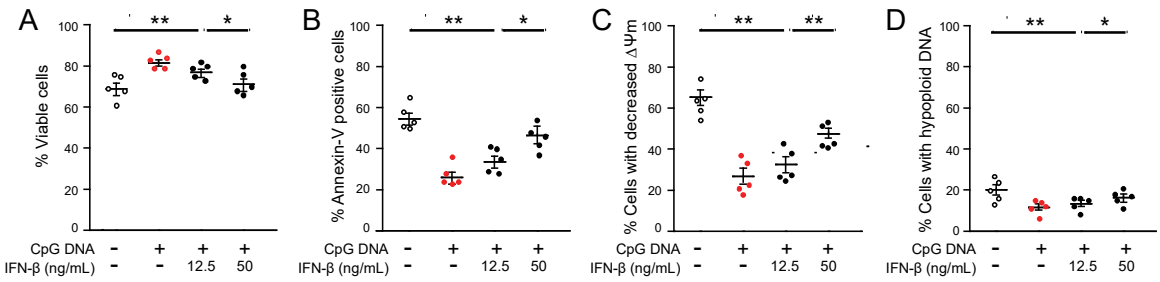

Fig. S1. IFN-β treatment reverses CpG DNA suppression of neutrophil apoptosis. Human neutrophils ($5x10^6$ cells/ml) were first challenged with CpG DNA (1.6 µg/mL) and then treated with IFN-β (12.5 or 50 ng/ml) 60 min later. Cell viability (propidium iodide staining) (**A**), annexin V staining (**B**), mitochondrial transmembrane potential ($\Delta\psi_m$, CMXRos staining) (**C**) and nuclear DNA content (**D**) were analyzed after 24 h of culture. Data are means ± SEM (n = 5 different blood donors). * P<0.05, ** P<0.01 (Dunn`s multiple contrast hypothesis test).

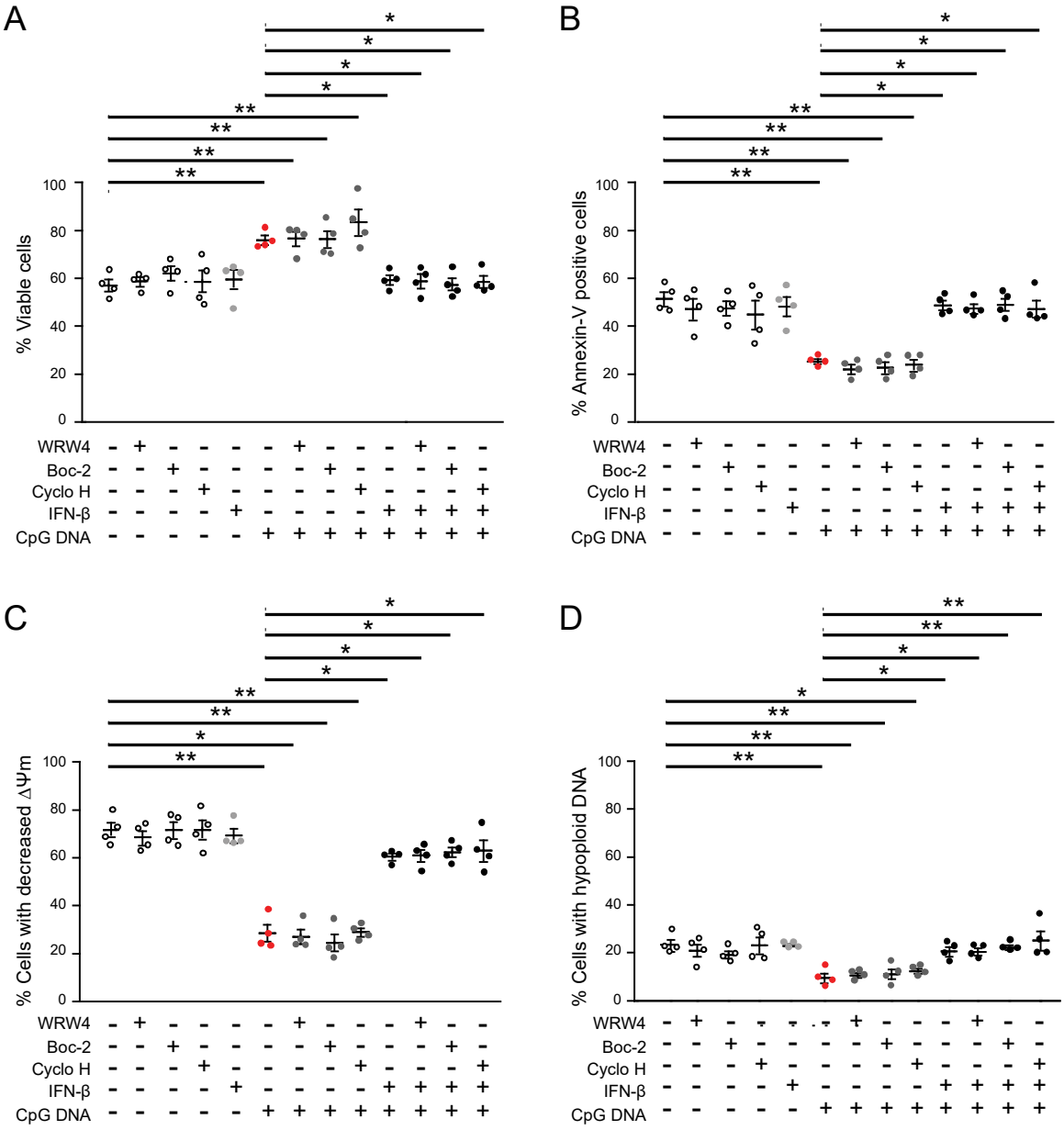

Fig. S2. FPR blockade does not affect the pro-apoptosis cues from IFN-β in neutrophils challenged with CpG-DNA. Human neutrophils ($5x10^6$ cells/ml) were cultured with IFN-β (50 ng/mL) in the absence or presence of the FPR1 antagonist cyclosporin H (1 μM) or the FPR2/ALX antagonist Boc-2 (50 μM) or WRW4 (5 μM) and then challenged with CpG DNA (1.6 μg/mL). Cell viability (propidium iodide staining) (**A**), annexin-V staining (**B**), mitochondrial transmembrane potential ($\Delta\psi_m$, CMXRos staining) (**C**) and nuclear DNA content (**D**) were analyzed after 24 h of culture. Data are means ± SEM (n = 4 different blood donors). * P<0.05, ** P<0.01(Dunn`s multiple contrast hypothesis test).

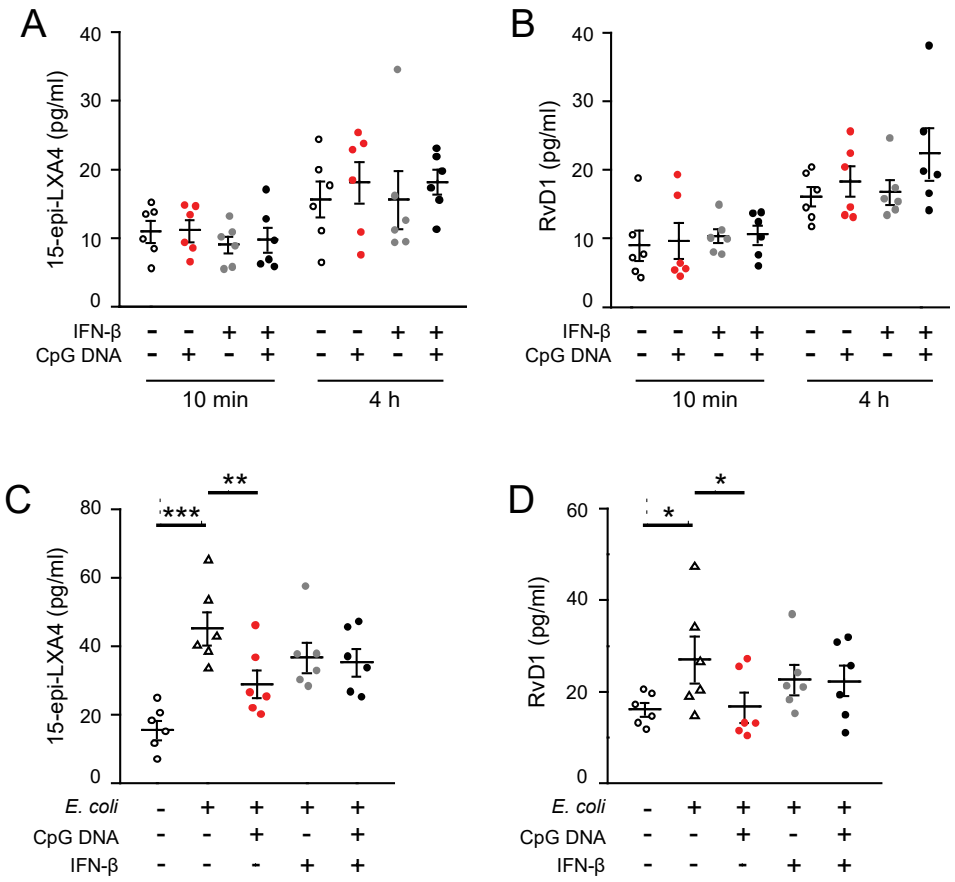

Fig. S3. Effects of IFN-β on 15-epi-LXA$_4$ and RvD1 secretion by neutrophils. Human neutrophils (10^7 cells/ml) were cultured with IFN-β (50 ng/mL) in the absence or presence of CpG DNA (1.6 μg/mL) for 10 min or 4 h. Conditioned media were collected and analyzed for 15-epi-LXA$_4$ (**A**) and RvD1 (**B**) by specific ELISAs. (**C, D**) Neutrophils were cultured in RPMI 1640 medium containing 10% autologous serum with CpG DNA (1.6 μg/ml) and *E. coli* at a ratio of 1:7 for 30 min, centrifuged, resuspended in RPMI 1640 culture media containing 2% autologous serum and challenged with IFN-β (50 ng/ml) for 4 h. Conditioned media were collected and analyzed for 15-epi-LXA$_4$ (**C**) and RvD1 (**D**) by specific ELISAs. Data are means ± SEM (n = 6 different blood donors). * P<0.05, ** P<0.01, *** P<0.001 (Dunn`s multiple contrast hypothesis test).

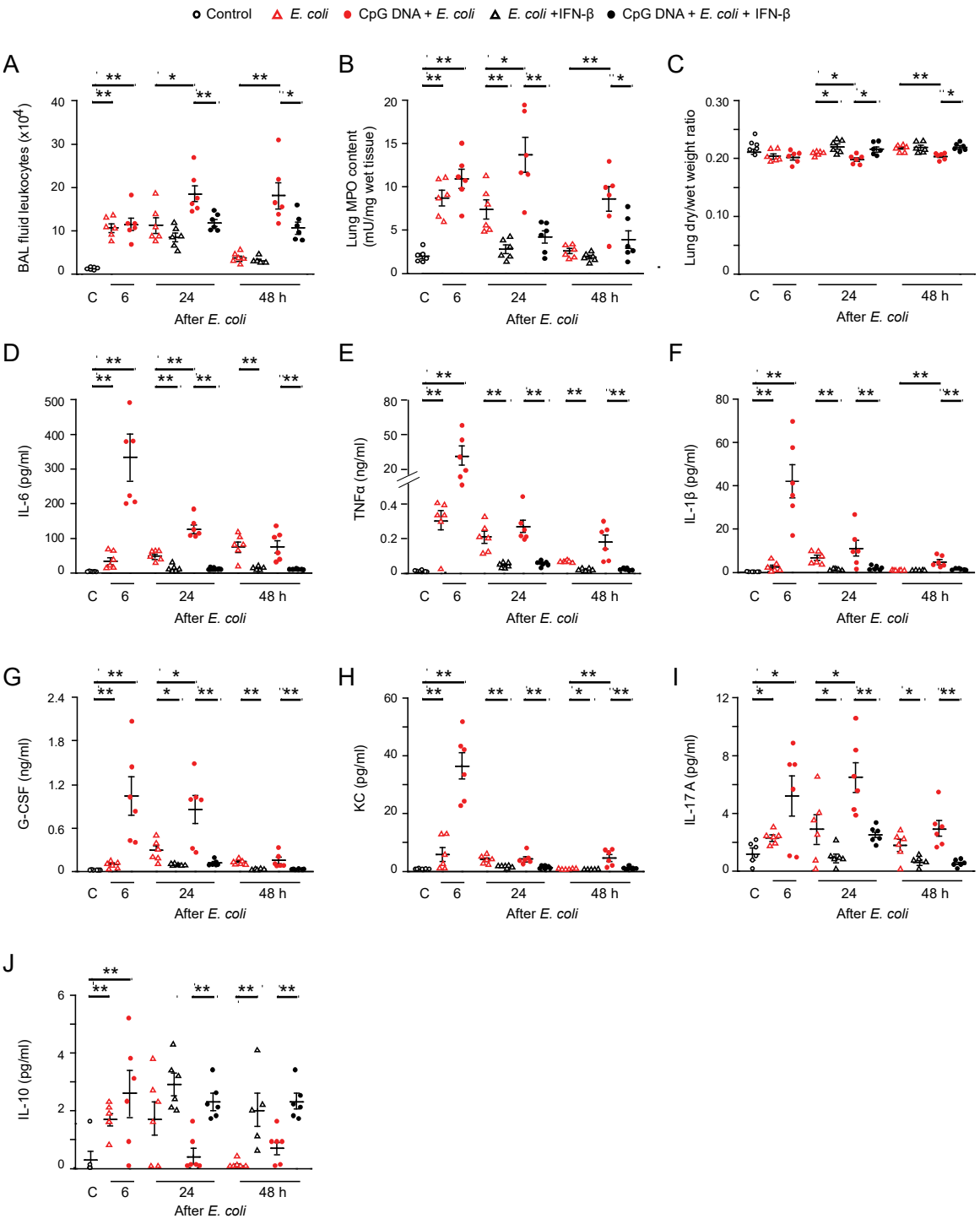

Fig. S4. IFN-β reduces pulmonary leukocyte accumulation, edema formation and BAL fluid pro-inflammatory cytokine levels. Acute lung inflammation was induced in female C57BL/6 mice by intratracheal instillation of $5x10^6$ live *E. coli* with or without

simultaneous injection of CpG DNA (1 μg/g b.w., i.p.). At 6 hours post *E. coli*, mice were treated with IFN-β (2.5 ng/g b.w., i.p.). Mice were killed at the indicated times, and bronchoalveolar lavage was performed or the lungs were processed for analysis without lavage. Lavage fluid total leukocyte counts (**A**), lung tissue MPO content (**B**), lung dry/wet weight ratio (**C**), lavage fluid levels of IL-6 (**D**), TNF-α (**E**), IL-1β (**F**), G-CSF (**G**), KC (**H**), IL-17A (**I**) and IL-10 (**J**) were determined. Results are means ± SEM (n = 6 mice per group). * $P<0.05$, ** $P<0.01$ (Dunn`s multiple contrast hypothesis test).

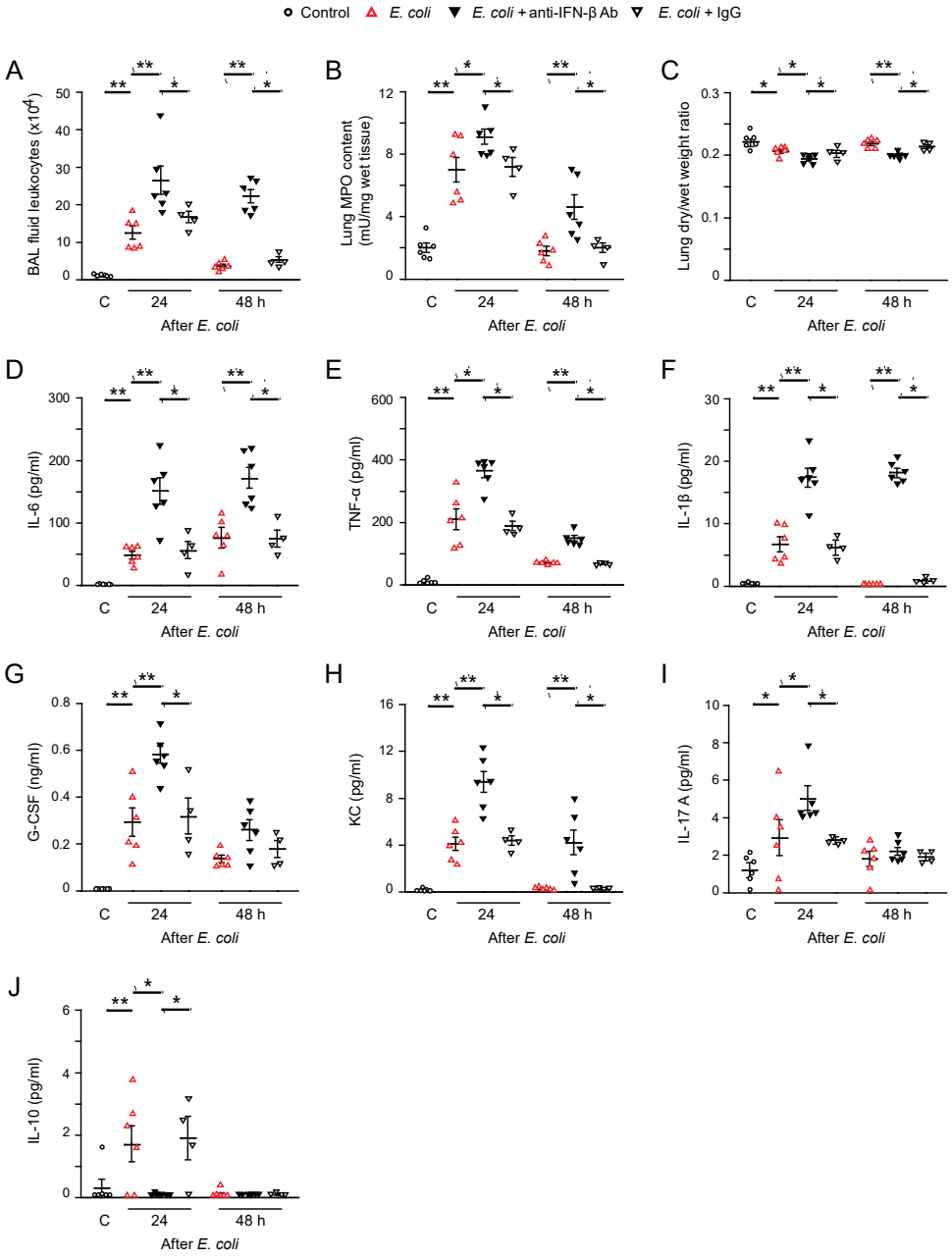

Fig. S5. Blockade of endogenous IFN-β aggravates leukocyte accumulation, edema formation and BAL fluid levels of pro-inflammatory cytokines. Female C57BL/6 mice was first injected with a rat anti-mouse IFN-β mAb or rat IgG (both at 50 ng/g b.w., i.p.) followed by intratracheal instillation of $5x10^6$ live *E. coli.* Mice were killed at the indicated times and bronchoalveolar lavage was performed or the lungs were processed for analysis without lavage. Lavage fluid total leukocyte counts (**A**), lung tissue MPO content (**B**), lung dry/wet weight ratio (**C**), lavage fluid levels of IL-6 (**D**), TNF-α (**E**), IL-1β (**F**), G-CSF (**G**), KC (**H**), IL-17A (**I**) and IL-10 (**J**) were determined. Results are means ± SEM (n = 4 mice for *E. coli* plus IgG; n = 6 mice per group for the other groups). * P<0.05, ** P<0.01(Dunn`s multiple contrast hypothesis test).

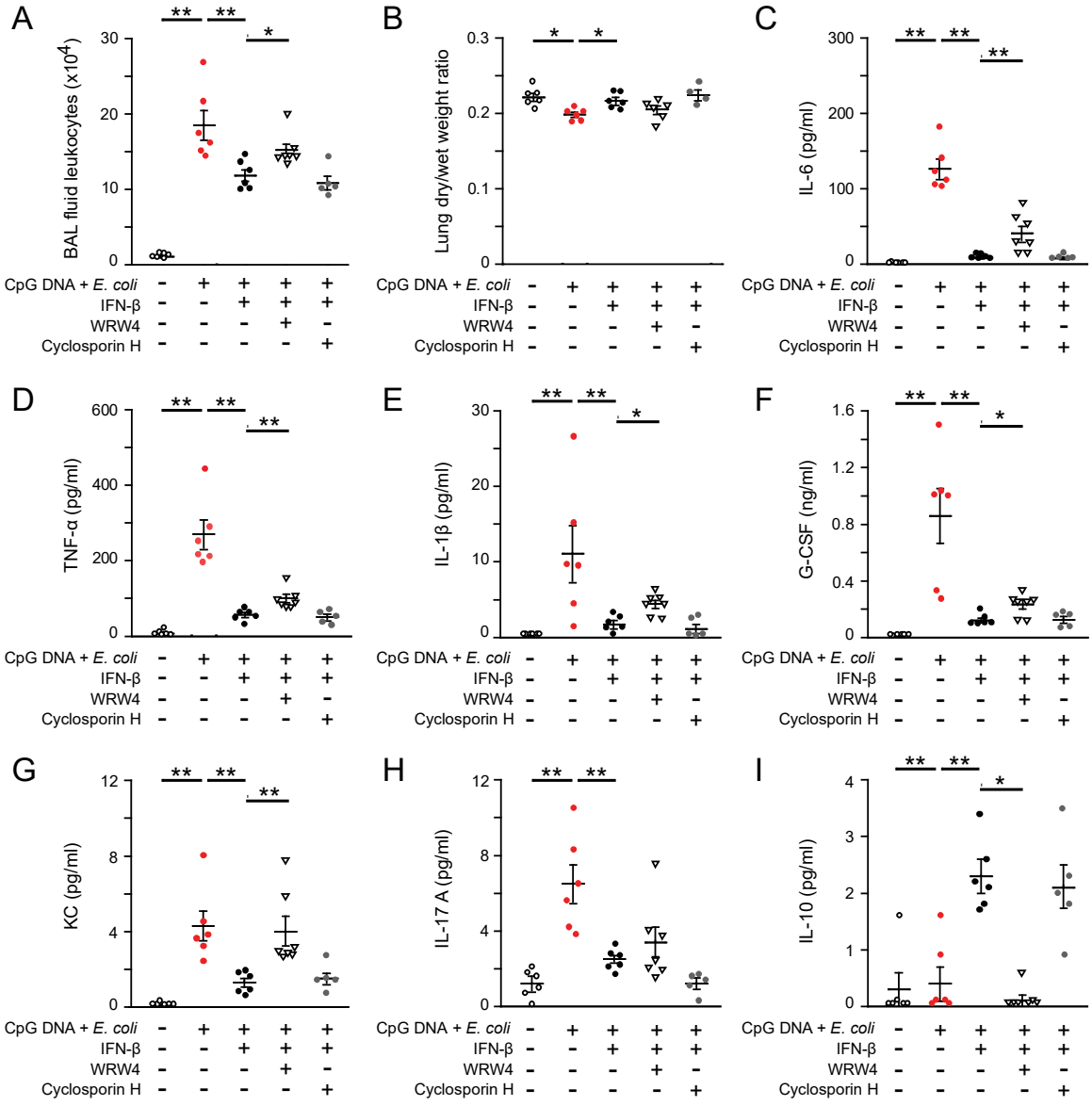

Fig. S6. Pharmacological blockade of ALX/FPR2 impairs IFN-β attenuation of pulmonary leukocyte accumulation and edema formation, and enhances lavage fluid pro-inflammatory cytokine levels. Acute lung inflammation was induced in female C57BL/6 mice by intratracheal instillation of $5x10^6$ live *E. coli* plus CpG DNA (1 μg/g b.w., i.p.). At 6 hours post *E. coli*, mice first received an i.p. injection of WRW4 (1 μg/g b.w.) or cyclosporin H (5 μg/g b.w.) followed by IFN-β (2.5 ng/g b.w., i.p.) 10 min later. Mice were killed at 24 h post *E. coli*, and bronchoalveolar lavage was performed or lungs were processed for analysis without lavage. Lavage fluid total leukocyte count (**A**), lung dry/wet weight ratio (**B**), and lavage fluid levels of IL-6 (**C**), TNF-α (**D**), IL-1β (**E**), G-CSF (**F**), KC (**G**), IL-17A (**H**) and IL-10 (**I**) were determined. Results are means ± SEM (n = 5-7 mice per group). * P<0.05, ** P<0.01 (Dunn`s multiple contrast hypothesis test).

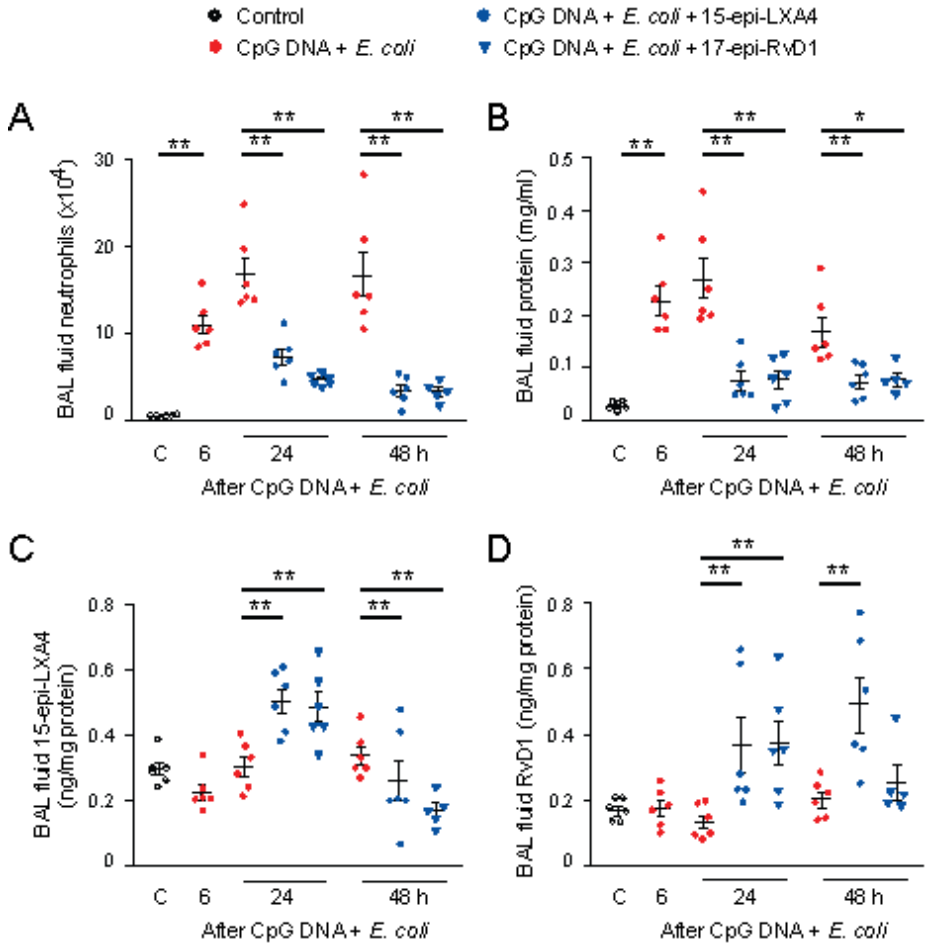

Fig. S7. 15-epi-LXA$_4$ and 17-epi-RvD1 enhance resolution of lung inflammation and trigger prolonged formation of pro-resolving lipid mediators. At 6 hours post induction of lung inflammation with intratracheal instillation of 5x10^6 CFU live *E. coli* plus CpG DNA (1 µg/g b.w., i.p.), female C57BL/6 mice were treated with either 15-epi-LXA$_4$ (125 ng/g b.w., i.p.) or 17-epi-RvD1 (25 ng/g b.w., i.p.). Mice were killed at the indicated times and bronchoalveolar lavage fluid neutrophil number (**A**), protein concentrations (**B**), 15-epi-LXA$_4$ levels (**C**) and RvD1 levels (**D**) were determined. Results are means ± SEM (n = 5-6 mice per group). * P<0.05, ** P<0.01 (Dunn`s multiple contrast hypothesis test).