Supplemental Information for

15-epi-LXA$_4$ and 17-epi-RvD1 restore TLR9-mediated impaired neutrophil phagocytosis and accelerate resolution of lung inflammation

Meriem Sekheri[a,b], Driss El Kebir[b], Natalie Edner[b] and János G. Filep[a,b,*]

[a]Department of Pathology and Cell Biology, University of Montreal, Montreal, QC, Canada H3T 1J4 and
[b]Research Center, Maisonneuve-Rosemont Hospital, Montreal, QC, Canada H1T 2M4

*Corresponding author: János G. Filep, MD
E-mail: janos.g.filep@umontreal.ca

www.pnas.org/cgi/doi/10.1073/pnas.1920193117

Materials and Methods

Bacterial and mitochondrial 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). Mitochondrial DNA (mtDNA) was isolated from human neutrophils ($2x10^7$) using Mitochondrial DNA Isolation kit (cat. no. K280, BioVision) in accordance with the manufacturer`s protocol, 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 (project 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. PMN ($5x10^6$ cells/mL, purity>95%, viability>98%, apoptotic<2%) were cultured in RPMI 1640 medium supplemented with 10% autologous serum on a rotator with neutrophil elastase inhibitor IV (NEI, 20 μM, Calbiochem), neutralizing anti-proteinase 3 antibody (5 μg/mL, clone WGM2, cat. no. ab91181, Abcam), cathepsin G inhibitor (CGI, 20 μM, Calbiochem),1,10-phenantroline (4 mM, Sigma-Aldrich), PMSF (2 mM, Sigma-Aldrich), and then challenged with CpG DNA or mtDNA(0.4-1.6 μg/mL). In some experiments, neutrophils were preincubated with 15-epi-LXA_4 (5S,6R,15R-trihydroxy-7E,9E,11Z,13E-eicosatetaenoic acid, 0.06-1 μM, Cayman Chemical) or 17-epi-resolvin D1(7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid, 12.5-200 nM, Cayman Chemical) with or without the FPR2/ALX inhibitor N-t-Boc-Phe-Leu-Phe-Leu-Phe (Boc 2, 50 μM, MP Biomedicals) or Trp-Arg-Trp-Trp-Trp (WRW4, 5 μM, Tocris) and then challenged with CpG DNA. Concentrations for 15-epi-LXA4 and 17-epi-RvD1 were informed from previous studies (2-5). In additional experiments, neutrophils were cultured with purified neutrophil elastase (0.001-1 μg/mL, Athens Research

&Technology) or proteinase 3 (0.03-3 μg/mL, Athens Research & Technology). At the designated time points, neutrophils were processed as described below.

CD11b and C5aR expression. Surface expression of CD11b and C5aR (CD88) on human freshly isolated neutrophils or on neutrophils challenged with CpG DNA (1.6 μg/mL) for 1 h was assessed using R-phycoerythrin-conjugated mouse anti-human CD11b Ab (clone D12, cat no. 347557, BD Biosciences) and FITC-labeled anti-CD88 (clone: S5/1, cat. no. 344306, BioLegend), respectively, with appropriately labeled isotype matched (IgG2a) irrelevant mAbs. 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 mAb.

ALX/FPR2 expression. Surface expression of FPR2 on human PMN challenged with CpG DNA (0.4 or 1.6 μg/mL) with or without *E. coli* at a ratio of 7 bacteria per cell for 1 h was assessed by staining with FITC-conjugated mouse anti-human ALX/FPR2 mAb (clone 30445, cat no. FAB3479F, R&D Systems). 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 of FITC-labeled, class-matched, irrelevant mAb.

Phagocytosis and phagocytosis-induced cell death. For quantitative analysis of phagocytosis, neutrophils were mixed 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. At the indicated times, the mixture was spun down to remove supernatant and the cells were resuspended in 100 μL ice-cold PBS containing 0.2% trypan blue to quench extracellular fluorescence (6). Intracellular fluorescence was analyzed with a FACSCalibur flow cytometer and CellQuestPro software.

To investigate apoptosis following phagocytosis, neutrophils were cultured with yeast (*S. cerevisiae* BY4741, 5 yeast particles per neutrophil) or *E. coli* (7

bacteria/neutrophil) with our without 15-epi-LXA_4 (1 μM), DPI (20 μM), TLR9 inhibitory ODN 5`tttagggttagggttaggg-3` (1.25 or 5 μM, cat. no. ODN TTAGGG, InvivoGen) (7), a negative control ODN 5`-tgctgctgcttgcaagcagcttgat-3` (5 μM, cat. no. ODN TTAGGG control, InvivoGen), CpG DNA or mtDNA (0.4-1.6 μg/mL). The yeast and *E. coli*ratios were chosen to avoid distortion of nuclear morphology by an overabundance of phagocytosed particles. Neutrophils were stained with acridine orange (10 μg/mL) and the percentage of cells with apoptotic nuclei (condensed or fragmented chromatin) was evaluated under an Olympus BX53 fluorescence microscope (6).

Apoptosis. Apoptosis in human PMN 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, 7). For analysis of nuclear DNA content, neutrophils were lysed and stained in 0.2 ml 0.1% sodium citrate solution containing 50 μg/mL propidium iodide and 0.1% Triton X-100 immediately before assay. Mitochondrial transmembrane potential was monitored following staining for 15 min with the lipophilic fluorochromechloromethyl-X-rosamine (CMXRos, 200 nM, Millipore-Sigma) and the fluorescence was analyzed in a FACSCalibur flow cytometer.

For analysis of caspase activation, at the indicated times the cell-permeable selective caspase-3 inhibitor FITC-labeled Asp-Glu-Val-Asp-fluoromethyl ketone (FITC-DEVD-fmk, 3 μM, BioVision) or the caspase-8 inhibitor FITC-labeled Ile-Glu-Thr-Asp-fluoromethyl ketone (FITC-IETD-fmk, 3 μM, BioVision) were added to PMN for 30 min and intracellular fluorescence was detected with flow cytometry (6).

Neutrophil elastase and proteinase 3. NE activity in conditioned media was measured with a colorimetric assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (2 mM, Sigma-Aldrich) as a substrate with purified human neutrophil elastase (Athens Athens Research & Technology) as standard (8). PR3 activity was measured with a colorimetric assay using Boc-Ala-Pro-Nva-4 chloro-SBzl (0.34 mM, Bachem) as a substrate in the presence of 5'5-dithio-bis-2-nitrobenzoic acid (0.17 mM) and purified human neutrophil PR3 (Athens Athens Research & Technology) as standard (9). PR3

activity was defined as the enzymatic activity that was inhibited in the presence of neutralizing anti-human proteinase 3 Ab (5 µg/mL).

Proteome Profiler Arrays. Human neutrophils (10^7 cells/mL) were challenged with CpG DNA (1.6 µg/mL) or vehicle for 30 min, lysed and proteins were quantitated using the human phospho-MAPK array kit (cat. no. ARY003B, R&D Systems) in accordance with the manufacturer protocol.

Murine lung inflammation. Female C57BL/6 mice (aged 8-14 weeks, Charles River Laboratories, St. Constant, QC, Canada) or *Tlr9*$^{-/-}$ mice (breeding pairs obtained from Dr. Akiko Iwasaki, Yale School of Medicine) were housed in pathogen-free conditions. The Animal Care Committee of the Maisonneuve-Rosemont Hospital approved the protocols for animal experiments and breeding (permit no. 2014-07 and 2015-31). Under isoflurane anesthesia, mice received intratracheal instillation of $5x10^6$ CFU live *E. coli* (American Type Culture Collection, ATCC 25922) in 50 µL saline with or without simultaneous intraperitoneal injection of CpG DNA (1 µg/g b.w. in 150 µl sterile saline). In separate groups of mice, first lung inflammation was induced by intratracheal instillation of live *E. coli*plus intraperitoneal injection of CpG DNA, followed 6 hours later (at near the peak of inflammation) by intraperitoneal injection of 15-epi-LXA_4 (125 ng/g b.w.) or 17-epi-RvD1 (25 ng/g b.w.) in 150 µl saline or appropriately diluted ethanol as a vehicle control. The doses for 15-epi-LXA4 and 17-epi-RvD1 were informed from previous studies (3, 10-12).

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). Bronchoalveolar lavage (BAL) fluid protein, and total and differential leukocyte counts were determined using standard techniques (3). Apoptosis in neutrophils (identified as Ly6G-positive cells) was assessed using flow cytometry with FITC-conjugated annexinV (BD Biosciences).The percentage of macrophages containing apoptotic bodies was assessed following staining with hematoxylin and eosin (3). BAL fluid cytokine levels were measured using a mouse multiplexed bead-based immunoassay (Bio-Plex Pro™

Coupled magnetic beads, cat. no. 171304070M, Bio-Rad) in accordance with the manufacturer`s protocol. BAL fluid levels of 15-epi-LXA_4 and RvD1 were measured using 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.

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 MPO (cat. no. M6908, Sigma) as a standard (3). Lung bacteria levels were assessed by growth on tryptic agarose using aliquots of lung homogenates.

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 or by ANOVA using ranks (Friedman test) followed by the Wilcoxon-Wilcox test for repeated measures. A value of $P<0.05$ was considered statistically significant. Graph Pad Prism 5 was used to calculate statistical differences.

References

1. L. József, T. Khreiss, J.G. Filep, CpG motifs in bacterial DNA delay apoptosis of neutrophil granulocytes. *FASEB J.* **18**, 1776-1778 (2004).

2. J.G. Filep, C. Zouki, N.A. Petasis, M. Hachicha, C.N. Serhan, Anti-inflammatory actions of lipoxin A_4 stable analogs are demonstrable in human whole blood: Modulation of leukocyte adhesion molecules and inhibition of neutrophil-endothelial interactions. *Blood* **94**, 4132-4142 (1999).

3. D. El Kebir *et al*., 15-epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury. *Am. J. Respir. Crit. Care Med.* **180**, 311-319 (2009).

4. Y.P. Sun *et al*., Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation. *J. Biol. Chem.* **282**, 9323-9334 (2007).

5. C.N. Serhan *et al*., Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J. Exp. Med.* **192**, 1197-1204 (2000).

6. D. El Kebir, P. Gjorstrup, J.G. Filep, Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 14983-14988 (2012).

7. I. Gursel et al, Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J. Immunol.* **171**, 1393-1400 (2013).

8. E.P. McGreal *et al*., Inactivation of IL-6 and soluble IL-6 receptor by neutrophil derived serine proteases in cystic fibrosis. *Biochim. Biophys. Acta* **1802**, 649–658 (2010).

9. V. Witko-Sarsat *et al*., Proteinase 3, a potent secretagogue in airways, is present in cystic fibrosis sputum. *Am. J. Respir. Cell Mol. Biol*. **20**, 729-736.

10. N. Chiang, X. de la Rosa, S. Liberos, C.N. Serhan, novel resolvin D2 receptor axis in infectious inflammation. *J. Immunol*. **198**, 842-851 (2017).

11. A. F. Bento, R.F. Claudino, R.C. Dutra, R. Macron, J.B. Calixto, Omega-3 fatty acid-derived mediators 17(R)-hydroxy docosahexaenoic acid, aspirin-triggered resolvin D1 and resolvin D2 prevent experimental colitis in mice. *J. Immunol*. **187**, 1957-1969 (2011).

12. V. Brancaleone *et al*., A vasculo-protective circuit centered on lipoxin A_4 and aspirin-triggered 15-epi-lipoxin A_4 operative in murine microcirculation. *Blood* **122**, 608-617 (2013).

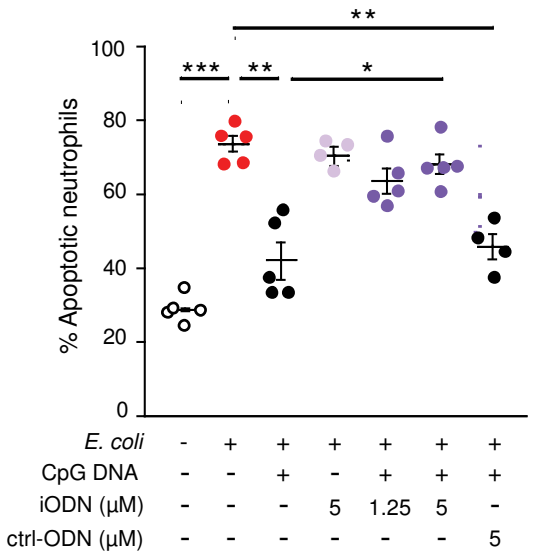

Fig. S1. CpG DNA and *E. coli* reduce surface expression of FPR2 on neutrophils. Human PMN ($5x10^6$ cells/mL) were cultured with CpG DNA with or without *E. coli* (7 bacteria per cell) for 60 min. Surface expression of FPR2 was assessed by flow cytometry. Results are means±SEM (n=5 different blood donors).*P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

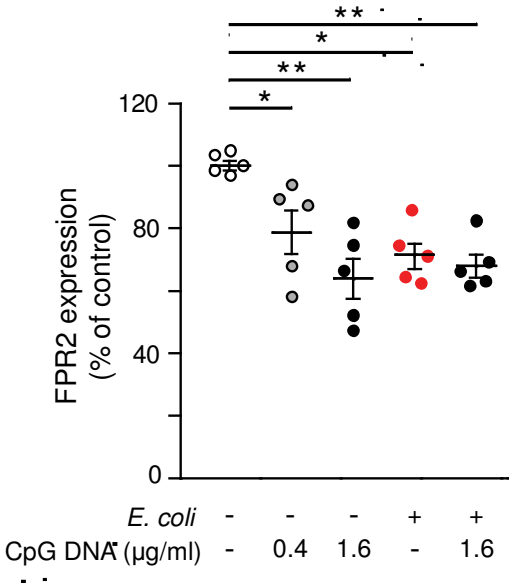

Fig. S2. Blockade of TLR9 prevents the anti-apoptotic action of CpG DNA in neutrophils. Human PMN ($5x10^6$ cells/mL) were cultured for 24 h with CpG DNA (1.6 µg/mL) and *E. coli* at a ratio of 1:7 with or without TLR9 inhibitory ODN (iODN) or irrelevant control ODN (ctrl-ODN). Apoptosis was assessed by nuclear morphology (condensed or fragmented chromatin) following staining with acridine orange (10 µg/mL). Results are means±SEM (n=4-5 different blood donors). *P<0.05, **P<0.01, ***P<0.001 (Dunn`s multiple contrast hypothesis test).

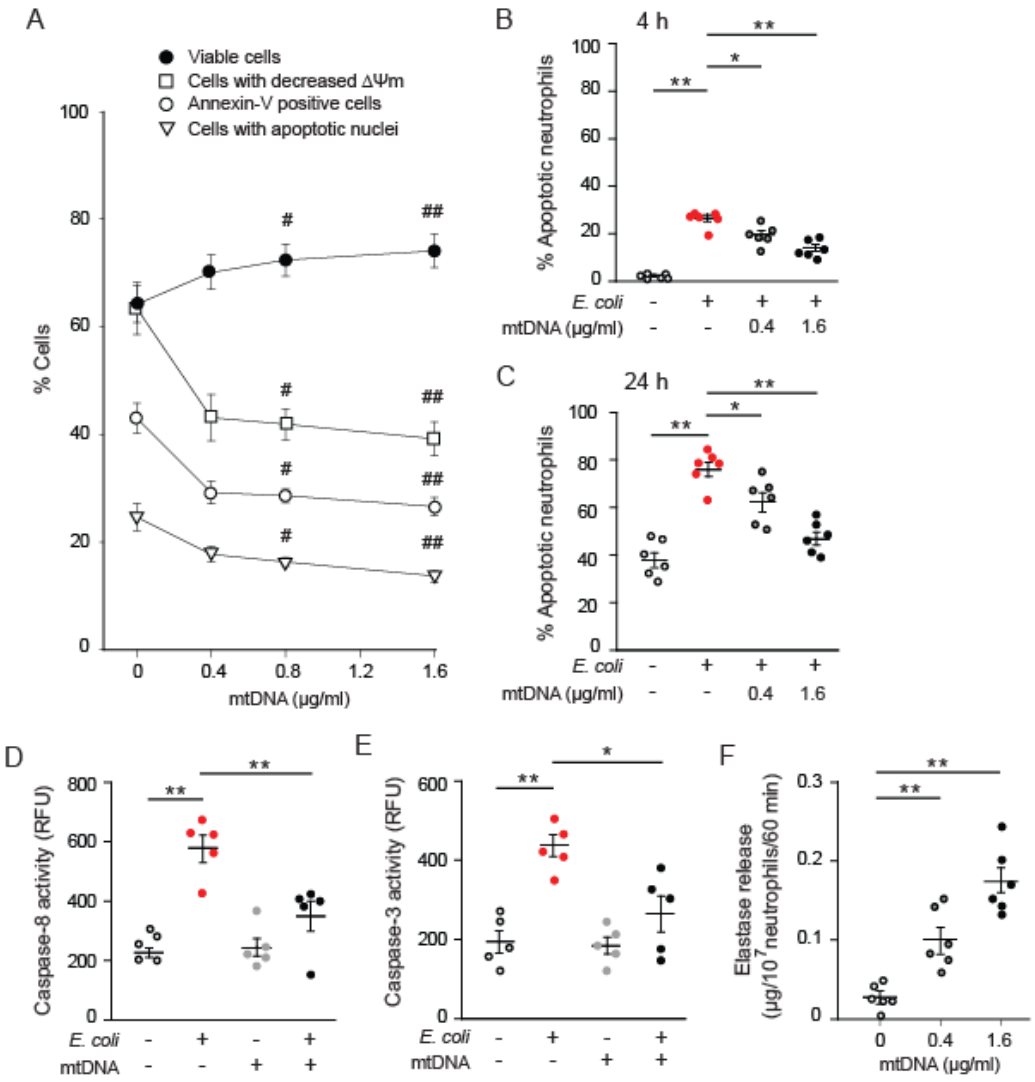

Fig. S3. Mitochondrial DNA impairs phagocytosis-induced neutrophil apoptosis. (**A**) Human PMN ($5x10^6$ cells/mL) were cultured with mitochondrial DNA isolated from human PMN for 24 h. Viability, mitochondrial transmembrane potential ($\Delta\Psi_m$) (CMXRos staining) and apoptosis (annexin-V-FITC binding and nuclear DNA content) were then assessed. Results are means ± SEM (n=6 different blood donors). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. vehicle (0 µg/ml) (Wilcoxon-Wilcox test). (**B-E**) PMN were challenged with mtDNA for 60 min then mixed with *E. coli* at a ratio of 7 bacteria per cell. Apoptosis was assessed by nuclear morphology at 4 h (**B**) and 24 h (**C**) following staining with acridine orange (10 µg/ml) under a fluorescence microscope. Results are means ± SEM (n=6 different blood donors). * P<0.05; ** P<0.01 (Dunn`s multiple contrast hypothesis test). Caspase-8 (**D**) and caspase-3 (**E**) activity was assessed at 4 h culture with flow cytometry using FITC-labeled Z-IETD-fmk and Z-FITC-DEVD-fmk, respectively. Results are means ± SEM (n=5 different blood donors). * P<0.05; ** P<0.01. (**F**) Elastase levels in the medium were assessed by a colorimetric assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as a substrate. Results are means ± SEM (n=6 different blood donors). **P<0.01 (Dunn`s multiple contrast hypothesis test).

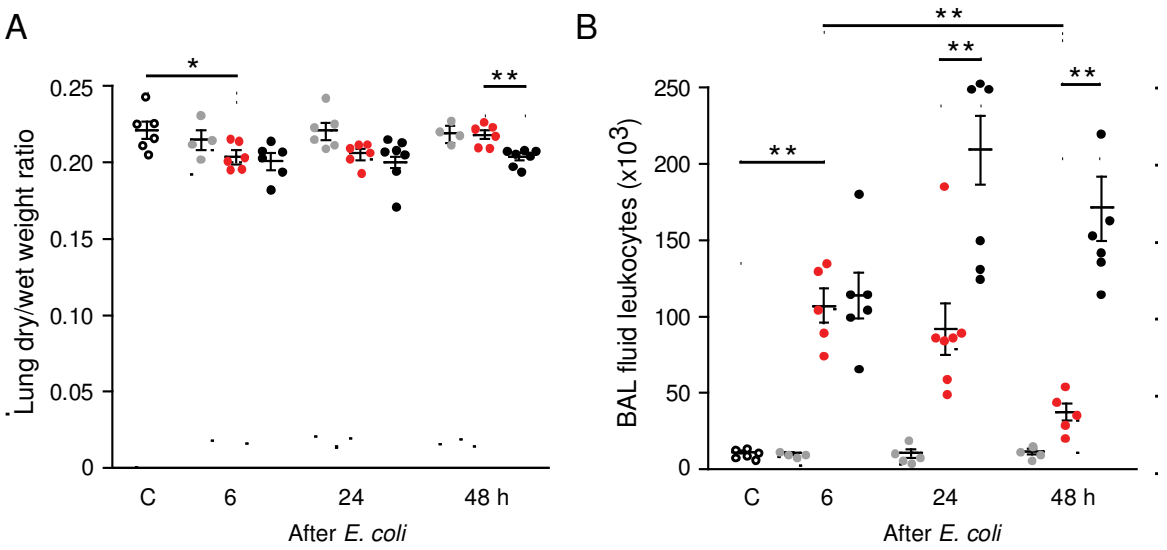

Fig. S4. **CpG DNA impairs resolution of *E. coli* pneumonia in mice.** Female C57Bl/6 mice were injected intratracheally with $5x10^6$ live *E. coli* with or without CpG DNA (1 µg/g b.w., i.p.). At 6, 24 or 48 h, the lungs were removed without lavage to assess dry/wet weight ratio (***A***) or subjected to BAL and total leukocyte counts were determined (***B***). Results are means±SEM (n=5-7 mice per group). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

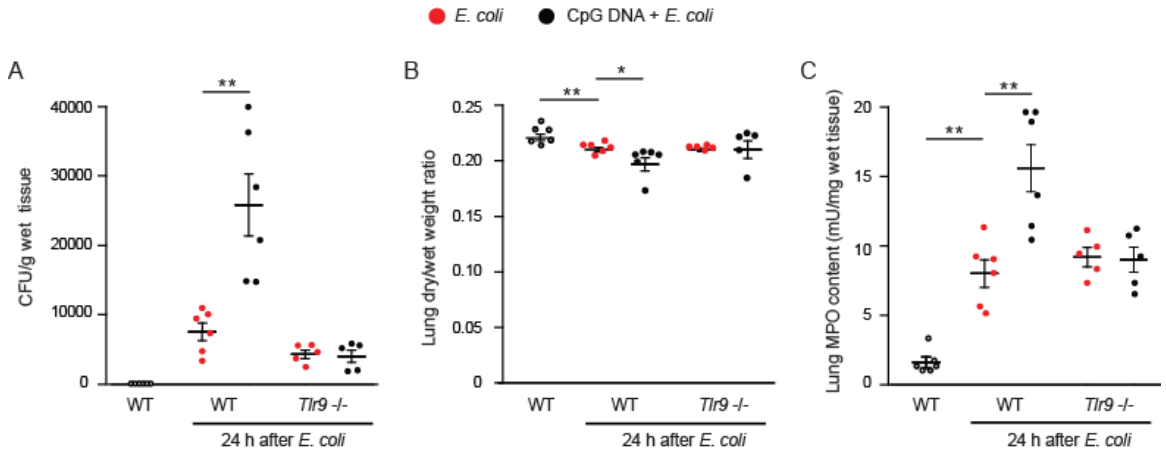

Fig. S5. Genetic deletion of TLR9 renders mice unresponsive to CpG DNA. Female wild type C57BL/6 and *Tlr9*$^{-/-}$ mice were injected intratracheally with 5x10^6 live *E. coli* with or without simultaneous intraperitoneal injection of CpG DNA (1 µg/g b.w.). The mice were euthanized at 24 or 48 h post-*E. coli*, and the lungs were removed without lavage and analyzed for *E. coli* content (***A***), lung dry-to-wet weight ratio (***B***) and tissue MPO activity (***C***). Naïve wild type mice served as control (WT). Results are means ± SEM (n=5-6 mice per group). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

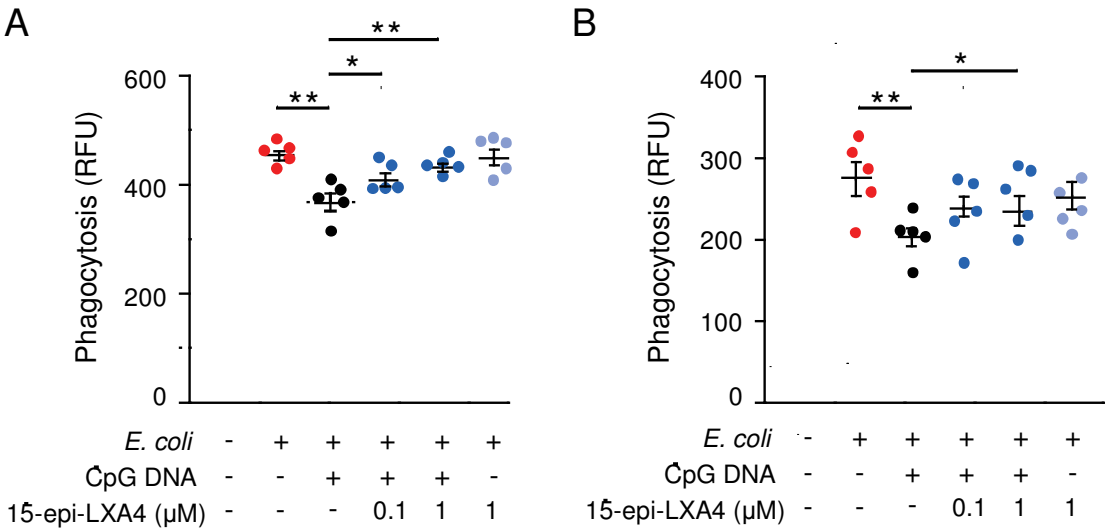

Fig. S6. 15-epi-LXA$_4$ enhances phagocytosis in blood neutrophils and monocytes challenged with CpG DNA. Human blood aliquots were cultured with 15-epi-LXA$_4$ for 10 min, CpG DNA for 60 min and then with opsonized FITC-labeled *E. coli* (7 bacteria per leukocytes) for 30 min. Following lysis of red blood cells, intracellular fluorescence in neutrophils (**A**) and monocytes (**B**) were analyzed with flow cytometry. Results are means±SEM (n=5 different blood donors). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

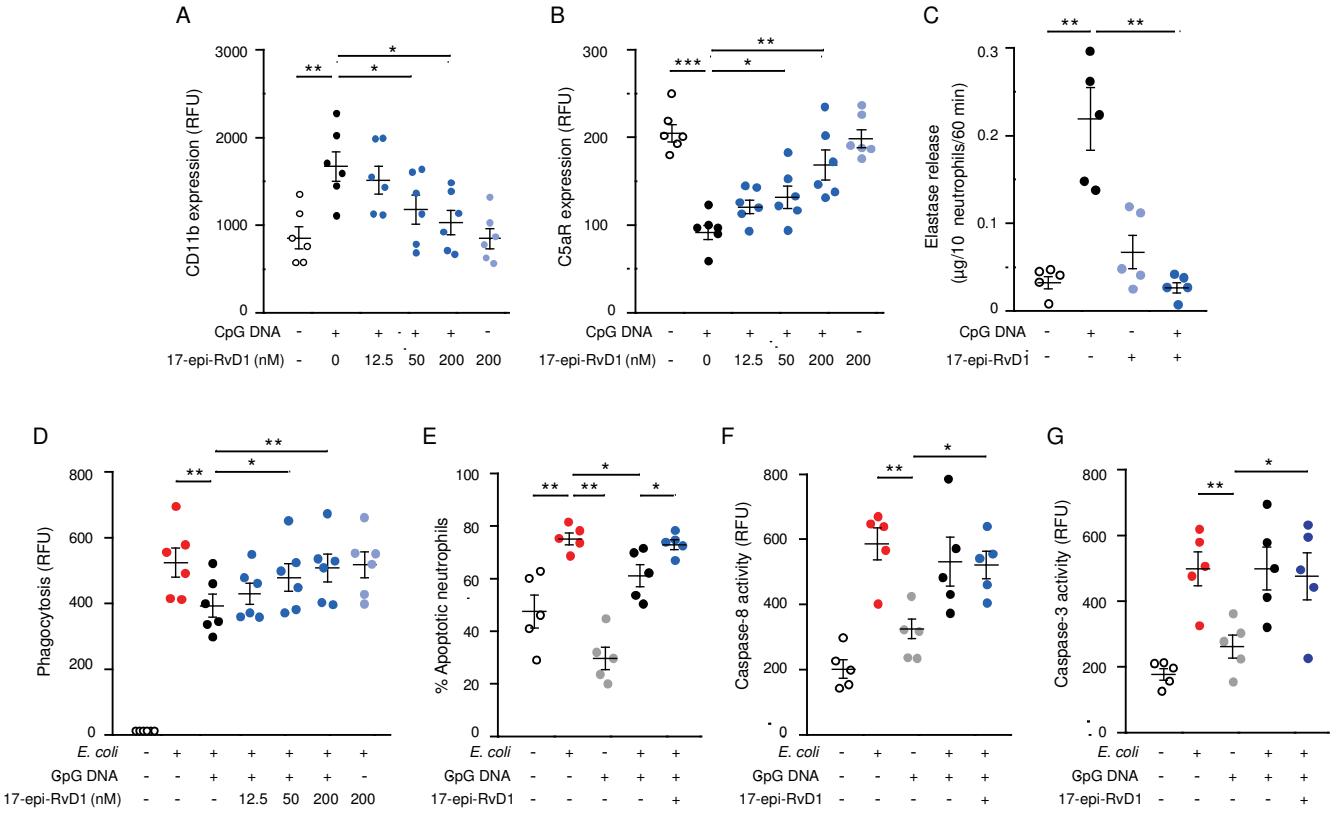

Fig. S7. 17-epi-resolvin D1 restores impaired phagocytosis-induced neutrophil apoptosis. Human PMN ($5x10^6$ cells/mL) were cultured for 10 min with RvD1 and then with CpG DNA (1.6 µg/mL) for 60 min. Surface expression of CD11b (***A***) and C5aR (CD88) (***B***) was assessed by flow cytometry, NEactivity was measured with a colorimetric assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as a substrate (***C***). Results are means±SEM (n=5-6 different blood donors).*P<0.05, **P<0.01, ***P<0.001. (***D***) PMN were cultured with 17-epi-RvD1 for 10 min, CpG DNA for 60 min and then with opsonized FITC-labeled *E. coli* (7 bacteria per neutrophil) for 30 min. Extracellular fluorescence was quenched with 0.2% trypan blue and intracellular fluorescence was analyzed with flow cytometry. Results are means±SEM (n=6 different blood donors). *P<0.05, **P<0.01. (***E-G***) Neutrophils were cultured with 17-epi-RvD1 for 10 min, followed by CpG DNA for 60 min and then with *E. coli* (7 bacteria per neutrophil). (**e**) Apoptosis was assessed by nuclear morphology at 24 h culture following staining with acridine orange (10 µg/mL). Results are mean±SEM (n=5 different blood donors). Caspase-8 (***F***) and caspase-3 (***G***) activity was assessed at 4 h culture with flow cytometry using FITC-IETD-fmk and FITC-DEVD-fmk, respectively. Results are means±SEM (n=5 different blood donors). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

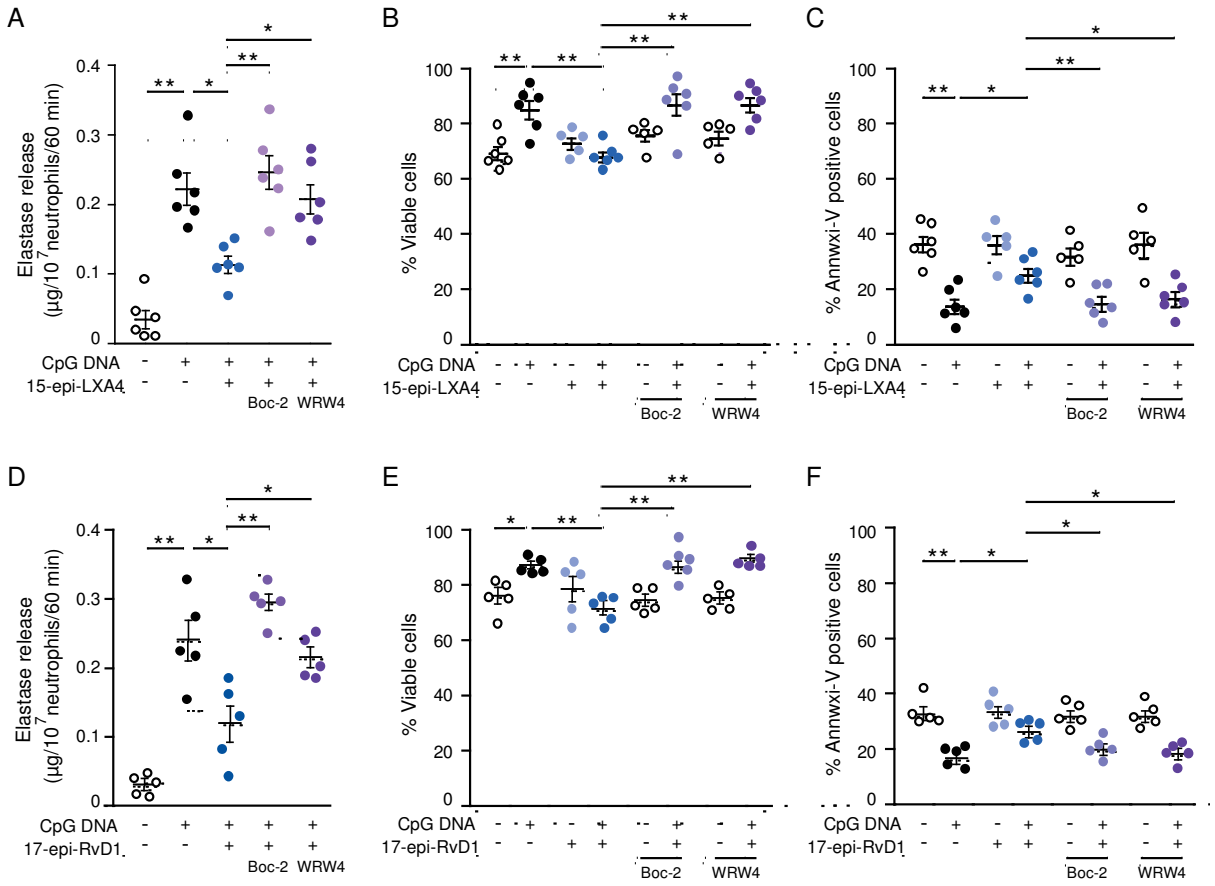

Fig. S8. Pharmacological blockade of FPR2 inhibits the actions of 15-epi-LXA$_4$and 17-epi-RvD1 on CpG DNA-stimulated elastase release and delay on neutrophil apoptosis. Human PMN (5x10^6 cells/ml) were preincubated with 15-epi-LXA$_4$ (1 µM) (***A-C***) or 17-epi-RvD1 (200 nM) (***D-F***) with or without Boc-2 (50 µM) or WRW4 (5 µM) for 20 min and then challenged with CpG DNA (1.6 µg/mL). (***A, D***) Elastase levels in the medium were assessed at 1 h post-CpG DNA by a colorimetric assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide as a substrate. Results are means ± SEM for 5-6 different blood donors. *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).Viability (***B, D***) and apoptosis (annexinV-FITC binding (***C, F***) were assessed at 24 h post-CpG DNA. Results are means ± SEM (n=5=6 different blood donors). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).

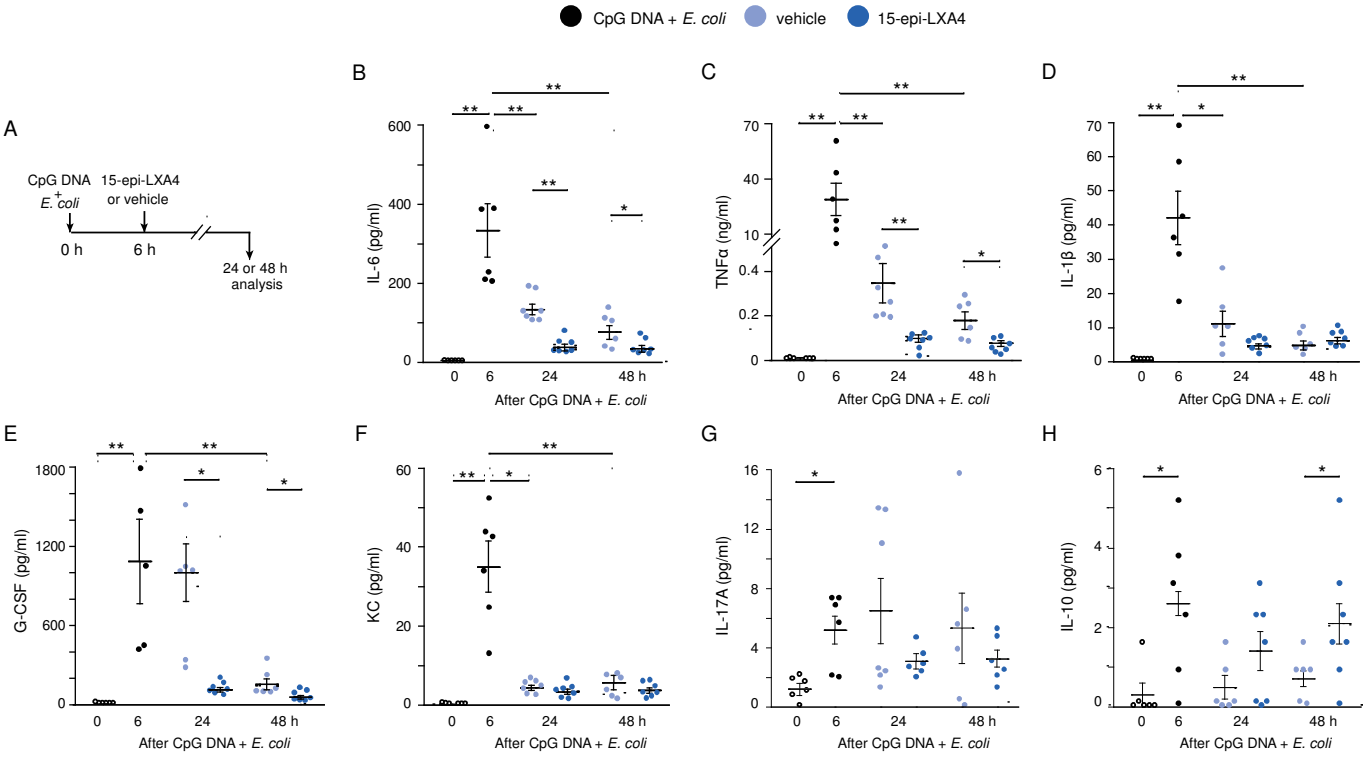

Fig. S9. BAL fluid cytokine profile in 15-epi-LXA$_4$ treated mice with *E. coli* plus CpG DNA-evoked pulmonary inflammation. (***A***) Female C57Bl/6 mice were treated with 15-epi-LXA$_4$ (125 ng/g b.w. in 150 µL saline, i.p.) or vehicle 6 h after intratracheal instillation of 5x10^6 live *E. coli* plus intraperitoneal injection of CpG DNA (1 µg/g b.w.). Mice were killed at the indicated times, and bronchoalveolar lavage fluid levels of IL-6 (***B***), TNFα (**C**) IL-1β (***D***) G-CSF (***E***), KC (***F***) , IL-17A (***G***) and IL-10 (***H***) were determined using a multiplex assay. Values are means±SEM (n=6-7 mice per group). *P<0.05, **P<0.01 (Dunn`s multiple contrast hypothesis test).