Supplemental Appendix

To accompany manuscript:

A Selective FPR2 Agonist Promotes a Pro-resolution Macrophage Phenotype and Improves Left Ventricular Structure-Function Post Myocardial Infarction

(Short title: Selective FPR2 Agonist & Cardiac Improvements Post-MI)

Supplemental Material and Methods

FPR2 knockout. FPR2 gene knockout mice were generated by homologous recombination targeting of C57BL/6N Tac ES cell line. Targeting vector was constructed with FPR2 gene fragment of BAC clones from the C57BL/6J RPCIB-731 BAC library. The FPR2 gene exon 2 (5'UTR and coding sequence from ATG to STOP codons) was replaced by an Flp-recombination-site-flanked positive selection cassette (neomycin resistance) which is expressed under the control of a eukaryotic promoter and contains a polyadenylation signal. The FPR2 3'UTR containing the polyadenylation site will be preserved in order to prevent any risk of transcriptional read through and putative splicing to downstream elements. Correctly targeted ES cell clones were selected by G418 resistance and screened by Southern blot analysis using probes from PCR primer pairs:

1) sense TCTGGGAATCTTAGGAAACCAAGAG/

antisense GAAGTGTCCATCACATATAACAGC;

2) sense GAAAATCAGGACAGTCTTAGGGTGC/

antisense GTTCTAACTGAGAGCCTGCATAGG.

After blastocyst injection and chimera breeding, founder mice were identified by PCR analysis using the following genotyping primers: Genotyping primers

sense GAAACCACACTTATACATTAACAAGG/

antisense GAAGTGGAATGAAAACCATCC would detect a 391 bp PCR product of the knockout allele.

Primers sense GTTTCCCTTTCAGCTTGTGG/

antisense GAAGTGGAATGAAAACCATCC would detect a 32 bp PCR product of the wild type allele.

Compound synthesis and formulation. BMS-986235 1-((3S,4R)-4-(2,6-difluoro-4-methoxyphenyl)-2-oxopyrrolidin-3-yl)-3-phenylurea, was synthesized as described (1). BMS-986235 was formulated in suspension vehicle composed of 0.5% carboxymethylcellulose in de-ionized water. Stock suspensions were made by vortexing the compound vigorously with glass beads and sonicating for 20 min. Aliquots for daily use were made and stored at -20°C. Thawed suspensions were vortexed vigorously before administration. Dosing suspension concentration was calculated assuming 100% active ingredient.

Acute lipopolysaccharide (LPS) rodent studies. Mouse studies were carried out at the Bristol Myers Squibb Lawrenceville, New Jersey site and rat studies were carried at the Biocon Bristol Myers Squibb Research Center in Bangalore, India. Male C57BL/6 mice were purchased from the Jackson Laboratory and were 10 to 12 weeks of age at the time of experiment. Male Sprague-Dawley rats (Taconic strain) were 8 to 10 weeks of age and obtained from Vivo Bio, Hyderabad, India. Rodents were randomized by body weight and pre-treated by oral gavage with BMS 986235 or vehicle (0.5% carboxymethylcellulose in water). One hour post dosing, mice and rats from all groups were challenged intraperitoneally (IP) with 0.3 mg/kg LPS (Sigma # L2630, St. Louis, MO) in sterile saline. Blood was taken 1 hr post LPS challenge from mice and 2 hr post LPS challenge from rats. Blood was processed by centrifugation to obtain plasma. Plasma IL-10 levels were measured by ELISA using kits specific for mouse (BD Biosciences #555252, Franklin Lakes, NJ) and rat (BD Biosciences #555134, Franklin Lakes, NJ).

Mouse myocardial infarction studies. Male C57BL/6 mice were purchased from the Jackson Laboratory and were 10 to 12 weeks of age at the time of surgery. Myocardial infarction (MI) was induced by permanent ligation of the left anterior descending (LAD) coronary artery using two procedures. For 28-day studies (UCSD), mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (8 mg/kg) given via the intraperitoneal route followed by oral intubation with a modified endotracheal tube. Mice were placed in a right lateral position on a circulating-water heating pad for maintenance of normal body temperature and mechanically ventilated at a tidal volume of ~0.5 ml and ~105 respirations per min. The neck and chest areas were shaved and prepped with 70% isopropyl alcohol followed by betadine solution. After draping for aseptic surgery, the heart was exposed via a left thoracotomy in the fourth intercostal space, and the pericardium incised. The descending left coronary artery was regionally located (*ramus interventricularis paraconalis*). Animals were randomized into treatment groups. The coronary artery was permanently ligated with a silk suture with a tapered curved 6-mm needle. Complete coronary occlusion was confirmed visually by noting the prompt and sustained pallor of the anterior wall distal to the ligation site. For animals designated as sham, the snare was placed but not tightened. The chest was closed in layers and the pneumothorax evacuated. For pain management, a local anesthetic (bupivacaine) was infiltrated subcutaneously along the edges of the incision sites, and buprenorphine (0.05 to 0.1 mg/kg) administered immediately postoperatively. Animals were returned to their cage, given water and standard rodent chow ad libitum, and monitored daily until the terminal procedure. For 3-day studies (Bristol Myers Squibb), mice were infarcted using a rapid method without the need of artificial ventilation during the surgery. Mice were anesthetized with 2% to 2.5% isoflurane inhalation and the chest cavity was exposed at the 4th intercostal space; the heart was exposed and moved through the

incision site by gently squeezing the chest cavity. The coronary artery was ligated using a 6-0 silk suture. Successful artery ligation was verified by noting the prompt and sustained pallor of the anterior wall distal to the ligation site. The heart was immediately repositioned in the chest cavity, and the incision was closed. Animals were allowed to recover in a warm box and returned to their home cages. Pain management was as described for the 28-day procedure. Mice were dosed once daily with BMS-986235 or with vehicle for 3 days or 28 days. Dosing was initiated 24 h post-MI. Dosing route was oral via gavage at a dose volume of 5 ml/kg. All dosing was carried out in the morning between 8 and 10 AM.

Rat myocardial infarction study (UCSD). Male Sprague-Dawley rats with body weight averaging approximately 235 g at the time of surgery were anesthetized with ketamine (50-100 mg/kg) and xylazine (5-10 mg/kg) cocktail via intraperitoneal injection. Rats were intubated with a 16 gauge catheter, placed on a mechanical ventilator and ventilated with room air. The fur on the left side of the chest was removed with a clipper and the skin was disinfected with a series of chlorhexidine, alcohol, and betadine scrubs according to standard aseptic procedure. The rat was placed in a supine position onto a surgical tray and a 2 cm skin incision was made at the level of the 4th and 5th intercostal space. The pectoral muscles were separated on the left side of the animal. A loose purse string pocket surrounding the incision was formed by using 3-0 vicryl suture with a taper needle (Ethicon, Cincinnati OH Cat #J311H) threaded through the skin and muscle layers. A hole was made in the intercostal space between the 4th and 5th ribs, and the heart was exposed via a small left thoracotomy. A 6-0 silk suture with taper needle was quickly inserted under the LAD coronary artery and tied tight with a double knot. The incision site was sutured closed in tissue layers. Sham operated animals underwent the same procedure without

coronary artery ligation. Post-surgical analgesia was administered during the recovery phase. Approximately 48 hr following MI, rats were evaluated by echocardiography (methods described in subsequent section) for impairment in systolic function. Rats having an ejection fraction of 50% or less were entered into the treatment-phase of the study. On the same day as the baseline echocardiography measurements, rats were sorted into treatment groups based on % ejection fraction in order to equalize the mean between groups. Immediately following grouping, rats were dosed with test article. Test article was given once daily to 5 days or 6 weeks after MI. Administration route was oral via gavage at a dose volume of 2 ml/kg. All dosing was carried out in the morning between 8 and 10 am.

Rat ischemia–reperfusion study (UCSD). Surgical preparation of male Sprague–Dawley rats was as described above in rat MI methods. Rats weighed ~225 g at the time of surgery. Following exposure of the heart, the region of the LAD coronary artery was identified (*ramus interventricularis paraconalis*) and transiently ligated (60 min). After release of the ligature, the chest wall and incision were sutured closed; animals were weaned from the respirator and allowed to recover. Sham-operated animals underwent the same procedure without coronary artery ligation. Post-surgical analgesia was given during the recovery phase. Rats were dosed once daily with BMS-986235 or with vehicle to 6 weeks after ischemia–reperfusion. Dosing route was oral via gavage at a dose volume of 2 ml/kg. All dosing was carried out in the morning between 8 and 10 am.

Echocardiography. Echocardiography was used to assess LV anatomy and systolic function. Long axis B-mode and short axis M-mode ultrasound was used to assess cardiac dimensions as

well as systolic function at designated times within the cardiac cycle, specifically at end of diastole and end of systole. Imaging was carried with a Vivid I echocardiographic system equipped with a 12 MHz linear i12L probe (GE Healthcare, Chicago, IL). A two-dimensional parasternal long-axis view of the LV was obtained in B-mode to define LV anatomy. The image window was changed to view the cross-sectional short axis of the LV using the papillary muscles as internal landmarks for image standardization. B-mode and M-mode was used to assess systolic function and LV dimensions via measurements of ejection fraction, LV wall thicknesses, LV chamber diameter and LV chamber area. Ejection fraction was measured from ultrasound data obtained at end diastole and end systole. The modified Simpson's method was used for ejection fraction calculations (2).

Ex vivo passive mechanics. Following exposure of the heart via thoracotomy, the heart was arrested in diastole by rapid intracardiac infusion of ice-cold cardioplegic solution containing high potassium and 2,3 butanedione monoxime. The heart was excised, the aorta cannulated and the heart perfused with cardioplegia to flush out residual blood. The atrial appendages were trimmed, the hearts weighed, and a cardiac balloon inserted into the LV across the mitral valve. The hearts were mounted onto a pressure–volume (PV) measurement stand and multiple epicardial surface markers placed onto the anterior wall. A digital camera was positioned to enable capture of video images of the anterior surface of the heart. PV curves were obtained by digitally acquiring left ventricular pressure and volume during balloon inflations. There were 2 to 3 conditioning runs followed by 2 to 3 data acquisition runs with maximal LV pressure of ~50 mmHg. Synchronized video images were obtained during each data run. Following acquisition of the PV data, the hearts were perfusion-fixed for 5 to 10 min by infusion of 10%

formalin into the aortic cannula while maintaining LV balloon pressure at 10 to 15 mmHg. Hearts were stored in 10% formalin at room temperature for subsequent histologic processing.

Flow cytometry. Hearts for flow cytometry were processed for cell isolations. Mice were injected intraperitoneally with 100 units of heparin and then anesthetized with 5% isoflurane. Hearts were perfused with 10 ml cold 1×PBS via the apex of the LV and removed from the chest wall. Connective tissue was removed and hearts were sliced in half along the long axis and rinsed in cold 1×PBS. Hearts were minced into small pieces (2 mm × 2 mm) and placed into a Miltenyi C Tube (Miltenyi Biotec, Germany) with 5 ml of digestion solution (472 units/ml collagenase, Type 4:1088 units/ml DNase 1 in HBSS+/+, 2% FBS, 0.2% BSA, 10 mM HEPES). Digestion into a single cell suspension was accomplished using the Miltenyi GentleMACS™ Octo 8 tissue dissociator. The digestion took 35 min to complete. The cell suspension was passed through a 40-µm cell strainer to remove tissue debris. Residual red blood cells were lysed with ACK Lysis Solution (Gibco Cat# A10492-01). Cell counts and viability were determined by acridine orange/propidium iodide staining on the Auto2000 cell counter (Nexcelom, Lawrence MA). For each sample, 2 x10^6 cells were stained with Live/Dead Aqua (Invitrogen Inc. Carlsbad, CA) according to manufacturer's instructions. Cells were then blocked for 15 minutes in 60 µl of Blocking Buffer (1XPBS-/- containing 3% fetal bovine serum, 5% mouse serum, 5% rat serum, 1% Fc Block, 10mM EDTA, 10mM HEPES, 0.1% sodium azide). Surface markers were then stained by addition of 60 µl of antibody cocktail containing 0.25 µg CD45[PerCP], Clone 30-F11, 0.25 µg Ly-6G[APC-Cy7] Clone 1A8, 0.75 µg CD64[BV421] Clone X54-5/7.1, 0.25 µg CD206[PE-Cy7] Clone C068C2, 0.25 µg MHC-II[PE] CloneAF6-120.1, 0.25 µg Ly-6C[FITC] CloneHK1.4, & 0.1 µg CCR2[APC] Clone 475301 in Blocking Buffer. After staining for 40

minutes on ice, cells were washed in 4 ml FACS Buffer (1XPBS-/- containing 3% fetal bovine serum, 10 mM EDTA, 10mM HEPES, 0.1% sodium azide) centrifuged and resuspended in 300 µl FACS Buffer. Data was acquired on a Cytek DxP10 FACS Calibur (Cytek Inc, Fremont, CA). Compensation was performed using UltraComp antibody capture beads (eBioscience Inc, San Diego, CA). To set gates, Fluorescence Minus One (FMO) controls were performed for each antibody (Fig. S12). Data was analyzed using FlowJo v10 (FlowJo LLC, Ashland OR). All antibodies were purchased from BioLegend Inc, San Diego, CA. except CCR2 which was from Novus Biological, Littleton, CO.

See table below for antibody specifications.

Antibody	**Clone**	**Conjugate**	**Company**	**Catalog#**	**Conc. (mg/ml)**	**Dilution**
CD45	30-F11	PerCP	Biolegend	103130	0.2	1:96
Ly-6G	1A8	APC-Cy7	Biolegend	127624	0.2	1:96
CD64	X54-5/7.1	BV421	Biolegend	139309	0.2	1:96
CD206	C068C2	PE-Cy7	Biolegend	141720	0.2	1:96
MHC-II	AF6-120.1	PE	Biolegend	116408	0.2	1:96
CCR2	475301	APC	Novus Biological	FAB5538A	0.01	1:12
Ly-6C	HK1.4	FITC	Biolegend	128006	0.5	1:240
Live/Dead Aqua	N/A	N/A	Life Technologies	L34957	N/A	1:1000

Histology. Hearts were perfusion fixed via aortic retrograde perfusion with 10% buffered formalin while maintaining a perfusion pressure at 10-15 mmHg. Hearts were stored in formalin until histologic processing. Processed hearts were cut along the short axis at mid-ventricle for paraffin embedding. Sections were cut (5µ) and stained with Masson's trichrome or picrosirius red for analysis of collagen, scar area and cavity/myocardial wall dimensions. Analysis was performed using the HALO digital pathology software. Immunohistochemical staining of

myocardial infarction border zone with Mouse monoclonal [6E3F8] to MMP2 and Mouse IgG2a, kappa monoclonal [MG2a-53] as the Isotype control (Abcam, Cambridge, Mass.) was performed. Visualization was achieved with 3, 3' Diaminobenzidine (DAB) producing a dark brown color. Immunohistochemical analysis of IL-10 and CD206 co-localization was performed on formalin-fixed and paraffin-embedded sections after deparaffinization and antigen retrieval. Sections were probed with mouse anti IL-10(A-2) Alexa Fluor 594 sc-365858 (Santa Cruz Biotech, Dallas, TX) and Rabbit anti-MRC1 (CD206) polyclonal bs-4727R (BIOSS Antibodies, Woburn, MA) overnight 4C. Sections are probed again, after washing with Goat anti-rbt IgG-AF488 4030-30 (Southern Biotech, Birmingham, AL) and mounted with Fluoroshield mounting medium containing DAPI ab104139 (ABCAM, Cambridge, MA). Images were acquired on a fluorescent microscope. In-situ hybridization experiments were carried out to detect arginase-1 and FPR2 mRNA levels in the peri-infarct border zone of mouse hearts. Histology files were analyzed in a blinded manner using image analysis software. Additional in situ hybridization methods are provided below.

In situ hybridization. Formalin-fixed hearts embedded in paraffin were cut to produce 5 μm thick sections, mounted onto slides and air-dried. In situ hybridization was performed using the Affymetrix ViewRNA^{TM} sequential branched-chain-DNA amplification technology. Proprietary probe sets were designed for mouse Arginase1 (NM_007482, Cat# VB1-17389-01) and FPR2 (NM_008039, Cat# VB1-10261) by the manufacturer. Each probe set consists of a series of twenty oligonucleotide pairs that hybridize to each target RNA. Signal amplification and visualization was achieved by sequential hybridization to a series of branched DNA structures concluding with hybridization to a type-6 specific alkaline phosphatase probe. Fast Blue

substrate was applied for FPR2 detection. This was followed by hybridization to a Type-1 specific alkaline phosphatase probe and Fast Red substrate for Arginase 1 detection. The target mRNAs were visualized using a standard bright field microscope and scanned at 20X using eSlide ManagerTM (Leica Biosystems, Wetzlar, Germany) followed by quantitation using the HALO Digital image analysis program (Indica Labs, Corrales, NM).

Phagocytosis assay. Mice were injected intraperitoneally with 1 ml of 2% BioGel P100 solution (BioRad, Inc. Hercules, CA). After 4 days, the peritoneum was lavaged with PBS/2 mM EDTA. Residual BioGel particles were removed by passing the exudate through a 40-µm strainer. Cells were washed with PBS and then seeded into 96-well black polystyrene clear flat bottom plates at a density of 1.2×10^5 cells/well. After 90 min, non-adherent cells were removed by washing with PBS and the macrophages were incubated overnight in Macrophage-SFM media (ThermoFisher, Inc Waltham MA). Macrophages were treated with test compound for 15 min and then fed sonicated opsonized FITC-labeled zymosan (Molecular Probes, Eugene OR) at a ratio of 8 particles/cell for 45 min at 37°C. Cells were washed gently with ice-cold PBS-/-. Phagocytosis was measured on a SpectraMAX Gemini EM plate reader (Molecular Devices, Inc. San Jose, CA) in the presence of 0.25% Trypan Blue in order to quench the fluorescence of residual non-ingested FITC zymosan.

Oxidative burst assay. HL-60 parental cells or FPR1 and FPR2 knockout cell lines (3) were used for oxidative burst experiments**.** A volume of 100 µl of HBSS buffer containing 400 µM of luminol (Sigma Inc, Cat# A4685 St Louis, MO), was used to seed ~100,000 cells/well using 96-well Opti-Plates (Perkin Elmer Cat#6005290, Waltham, MA). Approximately 2 µl of 50X-

concentrated test compound was prepared in assay buffer (1×HBSS/20 mM HEPES/0.1% fatty acid free BSA/DMSO 0.2%) and gently mixed. After a 15-min incubation at room temperature, 3 µl of 1 mg/ml zymosan (opsonized in charcoal-stripped human AB serum) was added to a final concentration of 30 µg/ml. Luminol bioluminescence was recorded every 2.5 min for 60 min at 37°C using LMaxII 384 well plate reader (Molecular Devices, San Jose, CA); data were analyzed using data at the 30-min time point, which is in the dynamic range of the assay.

Chemotaxis assay. HL-60 cells were differentiated for 5 days in 1.2% DMSO. The assay medium was phenol red-free RPMI with 0.2% fatty acid free BSA. Approximately 1×10^6 cells were added to the upper chamber of a Transwell plate (Corning Inc. Tewksbury, MA. Cat #3387). Migration was induced by placing chemoattractant in the bottom chamber and the differentiated HL-60 cells in the top chamber. Following migration, differentiated HL-60 cells in the lower chamber (migrated fraction) were quantitated using a luminescence cell-viability assay (Promega, Inc, Madison, WI Cat#G7571). For "antagonist mode" experiments, cells were pre-incubated with BMS-986235 for 15 min and added to the top chamber. Recombinant serum amyloid A1 peptide (0.8 µM; SAA) was added to the bottom chamber and chemotaxis quantified.

Apoptosis assay. Venous blood (anticoagulated with sodium heparin, 50 U/ml) was obtained from healthy volunteers who had denied taking any anti-inflammatory medications for at least 2 weeks. PMN were isolated by dextran sedimentation followed by Hypaque-Ficoll density centrifugation as described (Nauseef, W.M 2007). Neutrophils, 1 ml at 5 x 10^6 cells/ml (purity 95%, viability 98%) in HBSS (-/-/-) supplemented with 10% autologous plasma were placed on a

rotator and pre-incubated for 15 min with 2 μM BMS-986235 (0.02% DMSO final conc.) and then challenged with SAA (10 μg/ml final conc.) for 24 hr at 37°C in 5% CO_2. Viable, apoptotic and dead/necrotic PMN populations were assessed by flow cytometry after staining with PE-labeled annexin V and propidium iodide according to manufacturer's instructions (Biolegend Inc. San Diego, CA.)

Human blood assay. Donors were healthy consenting volunteers and confirmed that they were not taking any medications that affect immune responses within the past month, including oral, intravenous, or injectable steroids or non-steroidal anti-inflammatory drugs. Venous blood was drawn using standard butterfly needles into heparin vacutainer tubes. Each concentration of BMS-986235 was tested in duplicate. Whole blood (1.2 ml) was added to 12 μl of BMS-986235 at 100X final concentration in 12-well culture dishes and incubated on a rotating platform (80 rpm) at 37ºC for 1 hr. The concentrations tested were 10 μM, 1 μM, 0.1 μM and 0.01 μM. The final concentration of DMSO at 10 μM BMS-986235 was 1% and decreased 10 fold with each serial dilution. After 1 hr incubation, 1 ml of treated blood was added to a TruCulture® (Myriad RBM, Austin, TX) null tube containing 2 ml of optimized leukocyte culture media and was incubated at 37ºC for another 4 hr. Samples were centrifuge at 500 x g for 10 min and supernatant removed by aspiration leaving approximately a 5 mm layer over the cell pellet to which 2 ml PAXgene® Blood RNA Reagent (PreAnalytics ® Qiagen Inc. Hilden, Germany) was added, mixed and transferred to a 15 ml centrifuge tube. Isolation of total RNA was done according to the manufacturer's instructions (Qiagen, Inc. PAXgene Blood RNA Kit Handbook Version 2). Total RNA was quantified by absorbance at 260 nm using a Nanodrop 8000™spectrophotometer (Thermo Fisher Inc. Waltham, MA). Complementary DNA (cDNA)

was generated from 800 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc. Foster City, CA) according to the manufacturer's instructions. Gene-specific transcript quantification was performed by real-time quantitative PCR (qPCR) on a BioRad CFX384 Real Time PCR Detection System (BioRad Inc. Hercules CA) using the following commercially available TaqMan human primer and probe sets (Applied Biosystems, Foster City CA). IL-10 (Hs00961622), IL-6 (Hs00174131), IL-8 (Hs00174103), MCP-1 (Hs00234140), TNFα (Hs00174128), FPR2 (Hs00265954) and for normalization of input RNA RPL30 (Hs00265497). All primer/probe sets used span exon-exon junctions. Data were analyzed using the comparative Ct method (2ΔΔCt).

BMS-986235 blood concentration. For the various rodent studies, non-fasted blood was collected for measurements of compound exposures. BMS-986235 levels were analyzed from isolated plasma or from dried blood spots. Blood was taken by open-chest cardiac puncture at designated times following the final dose. Approximately 0.5 ml of blood was collected in 2 ml tubes containing K2-EDTA. For dried blood spot analysis, a 10 µl aliquot of whole blood was spotted on a 1 x 1 inch piece of filter paper (PerkinElmer Health Sciences, Inc. Greenville, SC) and dried overnight at room temperature. For plasma isolation, blood was centrifuged at 2000 rpm, the top plasma layer was transferred to microtubes and stored at -80°C. BMS-986235 was analyzed from plasma or from filters by triple quadrupole mass spectrometry utilizing an AB Sciex 6500 and Waters Acquity UPLC. Serum protein binding assays were determined using rapid equilibrium dialysis with a 5 hour incubation and LC-MS/MS analysis. Standard curves were prepared by serial dilution in biological matrix to match that of the samples being analyzed for BMS-986235. Standards and quality control samples were prepared in duplicate. Acetonitrile was used for protein

precipitation in the presence of an internal control. The lower limit of quantitation for BMS-986235 was 0.250 nM.

Bret-based signaling. The plasmid constructs used in the BRET experiments were previously described (3). BRET-based biosensor assays were carried out using HEK293 cells transiently transfected with the plasmids encoding FPR1 or FPR2 and a given biosensor. HEK293 cells were cultured in DMEM supplemented with Penicillin-Streptomycin and 10% Fetal bovine serum. Two days before the BRET experiment, HEK293 cells were transfected with either FPR1 or FPR2 and the different BRET biosensor components (see below*), using linear polyethylenimine 25 kDa (PEI) at a PEI:DNA ratio of 3:1. Transfected cells were directly seeded in 96-well plates pre-treated with poly-D-lysine, at a density of 35,000 cells per well, and maintained in culture for the next 48 hr. BRET experiments were carried out using the following procedures: cells were washed once with Tyrode's buffer (137 mM NaCl, 1 mM $CaCl_2$, 0.9 mM KCl, 1 mM $MgCl_2$, 3.6 mM NaH_2PO_4, 5.5 mM glucose, 12 mM $NaHCO_3$, and 25 mM HEPES, pH 7.4) and 90 µl of Tyrode's buffer was added to each well. Cells were equilibrated in their new buffer at room temperature for at least 30 min. Following the equilibration period, 10 µl of 10X prolume purple coelenterazine was added to each well (final concentration of 2 µM). Test compounds were subsequently added to each well using the HP D300 digital dispenser (Tecan, Mannedorf, Switzerland) (12 singlicate concentrations) and cells were incubated with the compounds for 15 min at room temperature. BRET readings were then collected using a Synergy Neo Multi-Mode reader (Bio Tek Instruments, Winooski, VT) with BRET2 filters 410/80 and 515/30. The BRET signal was determined by calculating the ratio of the light emitted by GFP (515/30 nm) over the light emitted by the Rluc (410/80 nm). The ligand-induced change in

BRET signal was determined by subtracting the signal obtained in absence of ligand from the values obtained after incubation with the test compounds. Sigmoidal concentration-response curves were generated with those values using a 4-parameter logistic equation using Prism (GraphPad Software, San Diego CA), to determine EC_{50} of the different compounds.

*Biosensor components co-transfected with FPR1 or FPR2:

Gi1 biosensor: Gαi1-Rluc, Gβ1 and GFP-Gγ1

Gi2 biosensor: Gαi2-Rluc, Gβ1 and GFP-Gγ1

Gi3 biosensor: Gαi3-Rluc, Gβ1 and GFP-Gγ1

GoA biosensor: GαoA-Rluc, Gβ1 and GFP-Gγ1

GoB biosensor: GαoB-Rluc, Gβ1 and GFP-Gγ1

G12 biosensor: Gα12-Rluc, P115-GFP, Gβ1 and Gγ1

G13 biosensor: Gα13-Rluc, rGFP-PDZ-RhoGEF, Gβ1 and Gγ1

β-arrestin1 biosensor: β-arrestin1-Rluc and rGFP-CAAX

β-arrestin2 biosensor: β-arrestin2-Rluc and rGFP-CAAX

Supplemental Tables

Supplemental Table 1. EC_{50}values of cAMP response determined in Chinese hamster ovary cells expressing FPR2 or FPR1

Assay	EC_{50} (nM)
Human FPR2 cAMP	5.0
Human FPR1 cAMP	400
Mouse FPR2 cAMP	0.5
Mouse FPR1 cAMP	500
Rat FPR2 cAMP	1.5
Rat FPR1 cAMP	720

EC_{50} values of cAMP response determined in Chinese hamster ovary cells expressing FPR2 and FPR1. BMS-986235 was a potent activator of FPR2 Gi coupling resulting in lowering of cAMP levels through adenylyl cyclase inhibition. Human and mouse EC_{50} values for FPR2 and FPR1 were previously described (Asahina et al. 2020).

Supplemental Table 2. Rat myocardial infarction following permanent coronary artery ligation.

	Sham	Vehicle	BMS-986235
Heart Rate (bpm)	312 ± 25	308 ± 30	310 ± 15
MAP (mmHg)	106 ± 15	106 ± 16	106± 10
Stroke Vol (µl)	171 ± 42*	125 ± 31	120± 26
Cardiac Output (ml/min)	53 ± 14*	38 ± 9	37 ± 9
Ejection Fraction (%)	59 ± 10**	35 ± 9	44 ± 13*
LV EDV (µl)	296 ± 71*	378 ± 105	300± 82*
LV ESV (µl)	157 ± 55**	283 ± 94	200 ± 72**
LV PSP (mmHg)	123± 13	120 ± 15	119 ± 9
LV EDP (mmHg)	8 ± 2*	15 ± 8	14 ± 7
LV dP/dt_{max} (mmHg/s)	6904 ± 757	5281 ± 1130	5859 ± 707
LV dP/dt_{min} (mmHg/s)	-8196 ± 853*	-5443± 1647	-5450 ± 1047
IVRT (ms)	10 ± 1*	15 ± 3	14 ± 3

Rat myocardial infarction following permanent coronary artery ligation. Left ventricular hemodynamics data for all groups. Statistical comparisons using a Dunnett's vs. vehicle, *p<0.05; **p<0.01. Data are reported as mean ± standard deviation. Abbreviations: LV EDV: left ventricle end diastolic volume; LV ESV: left ventricle end systolic volume; LV PSP: left ventricle peak systolic pressure; LV EDP, left ventricle end diastolic pressure; LV dp/dt_{max}: maximal rate of left ventricular pressure rise; LV dp/dt_{min} maximal rate of left ventricular pressure drop; IVRT: isovolumic relaxation time.

Supplemental Table 3. Rat myocardial infarction following ischemia-reperfusion injury.

	Sham	Vehicle	BMS-986235
Heart Rate (bpm)	309 ± 24	316 ± 21	298 ± 23
Stroke Vol (µl)	239 ± 46**	177 ± 47	200 ± 62
Cardiac Output (ml/min)	74 ± 14*	56 ± 16	59 ±16
Ejection Fraction (%)	67 ± 16***	38 ± 13	57 ± 19**
LV EDV (µl)	384 ± 81	474 ± 122	381 ± 113*
LV ESV (µl)	181 ± 80**	329 ± 127	210 ± 106**
LV PSP (mmHg)	124 ± 18	123 ± 12	122 ± 13
LV EDP (mmHg)	5 ± 4	6 ± 4	5 ± 6
LV dP/dt_{max} (mmHg/s)	6991 ± 733	6985 ± 897	6516 ± 704
LV dP/dt_{min} (mmHg/s)	-8002 ± 1213*	-6710 ± 1335	-6388 ± 1129
LV Vol. at dP/dt_{max} (µl)	372 ± 82	464 ± 120	371 ± 101*
LV Vol. at dP/dt_{min} (µl)	174 ± 74**	317 ± 125	201 ± 102**
IVRT (ms)	11 ±1	12 ± 1	12 ± 2

Rat myocardial infarction following ischemia-reperfusion injury. Left ventricular hemodynamics data for all groups. Statistical comparisons using a Dunnett's vs. vehicle, *p<0.05; **p<0.01, p<0.001***. Abbreviations: LV EDV: left ventricle end diastolic volume; LV ESV: left ventricle end systolic volume; LV PSP: left ventricle peak systolic pressure; LV EDP, left ventricle end diastolic pressure;; LV dp/dt_{max}: maximal rate of left ventricular pressure rise; LV dp/dt_{min} maximal rate of left ventricular pressure drop; LV Vol.: left ventricle volume; IVRT: isovolumic relaxation time. Data are reported as mean ± standard deviation.

Supplemental Table 4. Compound concentration of BMS-986235 measured in blood.

Model	Time Post Dose (hr)	Dose (mg/kg)/Blood Concentration (nM)	Dose (mg/kg)/Blood Concentration (nM)	Dose (mg/kg)/Blood Concentration (nM)
Mouse LPS Challenge	1.5	0.03 / 2 ± 1	0.3 / 8 ± 2	3 / 112 ± 50
Rat LPS Challenge	3	0.1 / 15 ± 6	1/ 146 ± 42	10 / 1222 ± 529
Mouse MI – Early Inflammation	1	3 / 107 ± 63		
Mouse MI	2	0.3 / 3 ± 3	3 / 84 ± 71	
Rat MI	1	1 / 210 ± 72		
Rat I/R	2	1 / 92 ± 46		

Compound concentration of BMS-986235 measured in blood. Compound levels were measured at the designated times post dose in the various animal models. Blood samples were taken at the end of each respective study. In most cases, plasma was isolated for analysis of compound exposure with the exception of the Rat MI experiment, where a dried blood spot method was used (see Supplementary Methods in online appendix). Data are reported as mean ± standard deviation. Abbreviations: LPS: lipopolysaccharide; MI: myocardial infarction.

Supplemental Figure Legends

Supplementary Figure S1. Activation of G protein and β-arrestin recruitment following stimulation of rat FPR2 (rFPR2) and mouse FPR2 (mFPR2) with BMS-986235. Activation of rFPR2 **(A, C)** and mFPR2 **(B, D)** by BMS-986235 was assessed using BRET biosensors detecting Gαi2 (A, B) and β-arrestin1 (C, D). HEK293 cells expressed either rFPR2 or mFPR2. Data represent the mean ± SEM of three independent experiments.

Supplementary Figure S2. Cytokine gene expression in isolated human blood. (A) TNFα; **(B)** IL-8.

Supplementary Figure S3. Representative plots for apoptotic and viable isolated human neutrophils treated with SAA +/- BMS-986235. Cells are gated on neutrophil population by FSC vs SSC and doublets removed by FSC-A vs. FSW-W. Gates were set using fluorescence minus one (FMO) controls as shown in the figure.

Supplementary Figure S4. Representative histology images of mouse MI LV cross sections. (A) Masson's trichrome staining for measurement of collagen (blue stain); **(B)** Masson's trichrome staining for infarct area (light blue to white stain); **(C)** Immunohistochemical staining of MI with Mouse monoclonal [6E3F8] to MMP-2 (dark brown, white arrows) and Mouse IgG2a, kappa monoclonal [MG2a-53] - Isotype control.

Supplementary Figure S5. Representative in situ hybridization images from the peri-infarct border zone on day 3 post myocardial infarction in the mouse. Vehicle treatment vs. 3 mg/kg BMS-986235 treatment. Arginase 1 (red) and FPR2 (blue). Nuclear chromatin is stained

light blue with Gill's hematoxylin. These two images compare the contrastive effects of treatments on arginase 1 expression.

Supplementary Figure S6. Gating strategy. The gating and cell identification strategy is shown. The total leukocyte population is identified based on their expression of CD45. Live cells are then selected by exclusion of Live/Dead Aqua positive staining. Cell doublets are removed by FSC-A vs FSC-H gating. Neutrophils are then extricated by their expression of the neutrophil-specific marker Ly-6G. The monocyte/macrophage population is identified from the remaining leukocytes based on expression of CD64 (Fcγ Receptor I) and M1/M2 polarization determined based on expression of CD206 (mannose receptor).

Supplementary Figure 7. Absolute numbers of total leukocytes, neutrophils and macrophages in the heart following MI by flow cytometry. (A) Representative bivariate plot of final gate for CD206+ macrophage population. (B) Total CD45+ leukocytes, (C) total CD45+ leukocytes that are CD64+ monocyte/macrophages, (D) total CD64+ monocyte/macrophages that are CD206+, "M2," (E) total CD64+ monocyte/macrophages that are CD206-, "M1," (F) total CD45+ leukocytes that are Ly6G+ neutrophils. Treatments consisted of vehicle (n=7) or 3 mg/kg BMS-986235 (n=10). Non-infarcted sham hearts (n=10) are shown for comparison. T-test vs vehicle: *$p < 0.05$, **$p<0.01$, ***$p<0.001$.

Supplementary Figure S8. Immunohistochemistry of CD206 (green) and IL-10 (red) at the peri-infarct zone of anterior wall myocardial infarction in rats following permanent coronary artery occlusion. Rats were treated with BMS-986235 (1 mg/kg) or vehicle 48 hours post coronary artery ligation and dosing occurred once daily to 5 days post MI. Antibody for IL-10 detection was a mouse monoclonal labeled with ALEXAFLUOR 594. CD206 detection was

done with an unlabeled primary polyclonal followed by a FITC-labeled secondary antibody. Cell nuclei detection was carried out with DAPI (4′,6-diamidino-2-phenylindole) DNA-binding dye (blue). CD206-positive macrophages are the predominant IL-10 producing cells surrounding the infarct. For BMS-986235 treatment, an overlay of CD206 and IL-10 is provided. Images are shown with 200X magnification**.**

Supplementary Figure S9. Acute IL-10 response in rat. Blood IL-10 levels were measured in rats challenged with lipopolysaccharide. Rats were pre-treated with BMS-986235 or vehicle (PO gavage) 1 hr prior to challenge with IP-administered lipopolysaccharide 0.3 mg/kg. The chosen dose elicited a significant but non-lethal inflammatory response. Plasma samples were taken 2 hr post lipopolysaccharide-challenge. Three independent studies were pooled. Group sizes: n=24 per treatment. Dunnett's vs. vehicle, *$p < 0.05$; ***$p < 0.001$.

Supplementary Figure S10. Rat heart long axis echocardiography images. Figure depicts representative B-mode cardiac echocardiographic long axis images captured at end-diastole and end-systole. Groups are Sham (no MI), Vehicle +MI, and BMS-986235 + MI treated animals. Superimposed are LV cavity area outlines.

Supplementary Figure S11. Infarct wall thickness. The thickness of the mid-infarct wall was measured for infarcted rats treated with vehicle or BMS-986235 (1 mg/kg). A similar region was measured in non-infarcted rats that underwent surgical preparation but without tightening of the ligature ("shams").

Supplementary Figure S12. Fluorescence minus one (FMO) control. FMO controls were performed for each antibody in the panel to allow precise gate setting and proper identification and measurement of cell populations.

Supplemental Figures

Figure S1.

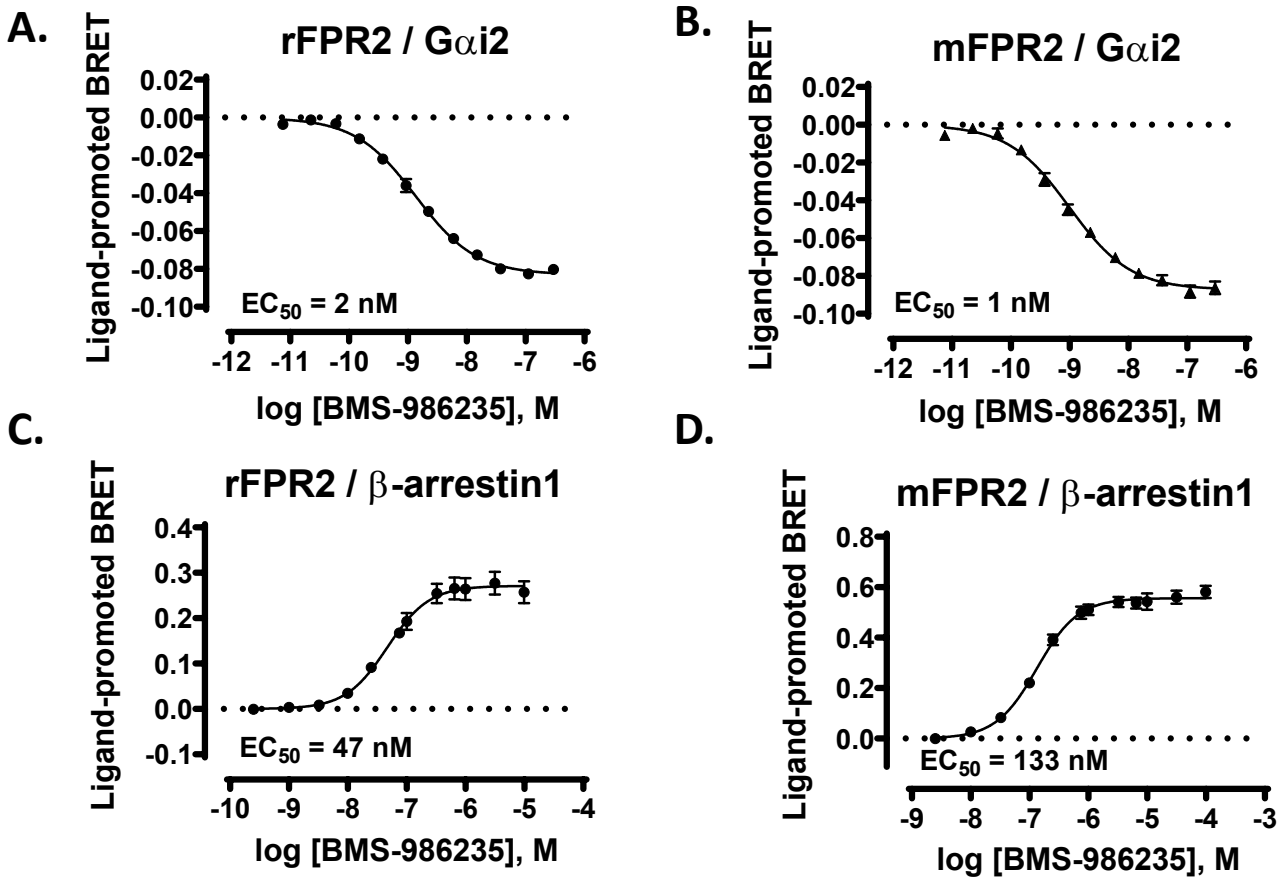


Figure S2.

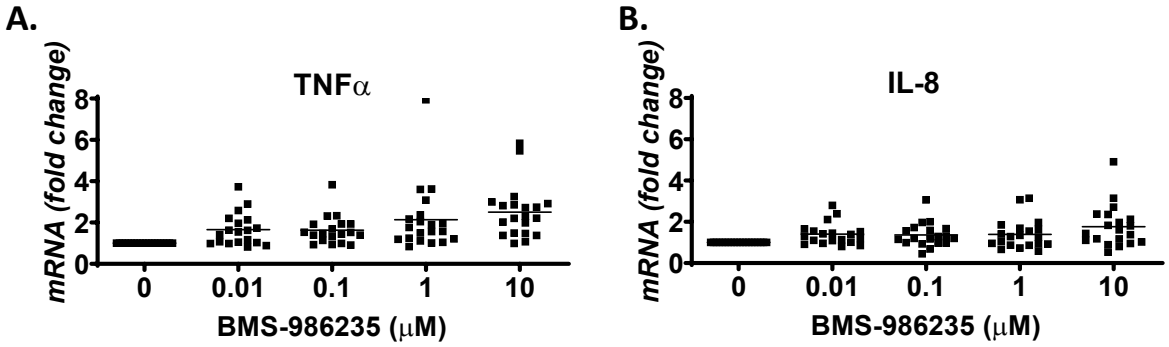

A.
TNFα
mRNA (fold change)
0
2
4
6
8
0
0.01
0.1
1
10
BMS-986235 (μM)
B.
IL-8
mRNA (fold change)
0
2
4
6
8
0
0.01
0.1
1
10
BMS-986235 (μM)

Figure S3.

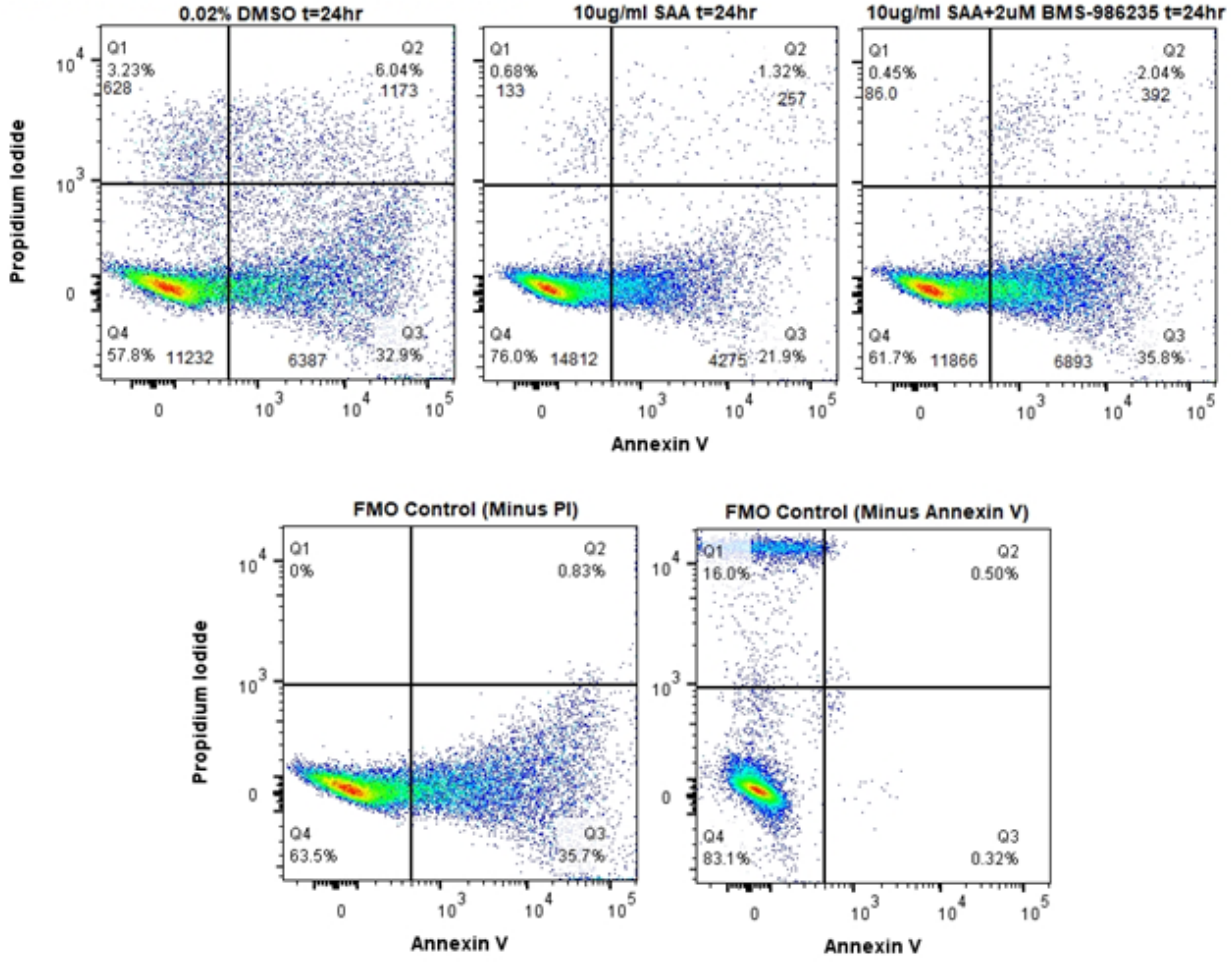

0.02% DMSO t=24hr
10ug/ml SAA t=24hr
10ug/ml SAA+2uM BMS-986235 t=24hr
Propidium Iodide
Annexin V
Q1 3.23% 628
Q2 6.04% 1173
Q4 57.8% 11232
Q3 32.9% 6387
Q1 0.68% 133
Q2 1.32% 257
Q4 76.0% 14812
Q3 21.9% 4275
Q1 0.45% 86.0
Q2 2.04% 392
Q4 61.7% 11866
Q3 35.8% 6893
FMO Control (Minus PI)
FMO Control (Minus Annexin V)
Q1 0%
Q2 0.83%
Q4 63.5%
Q3 35.7%
Q1 16.0%
Q2 0.50%
Q4 83.1%
Q3 0.32%

Figure S4.

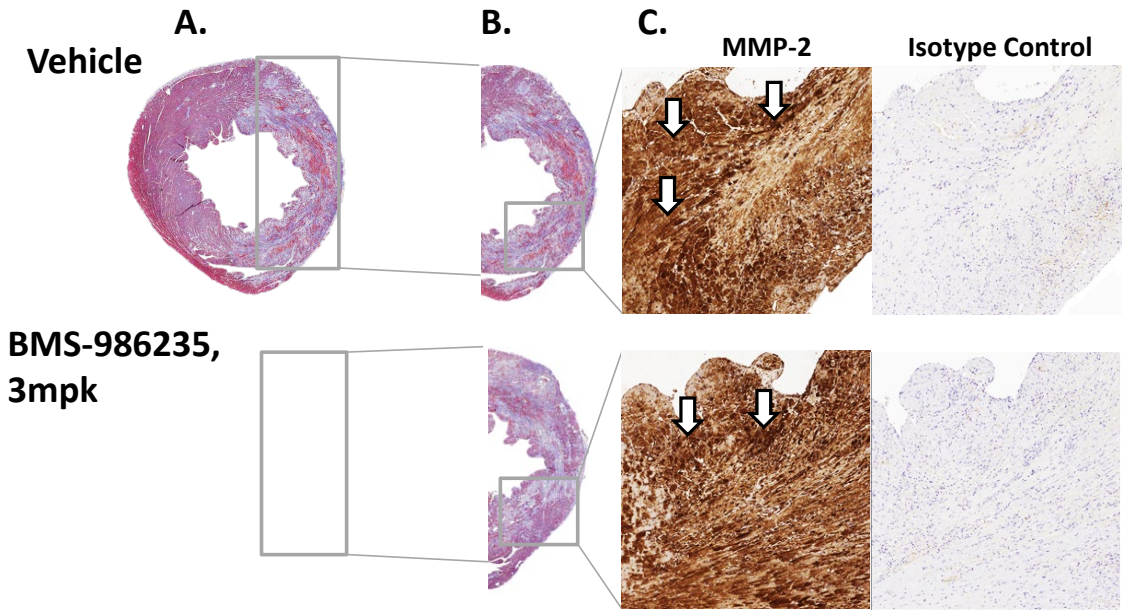


Figure S5.

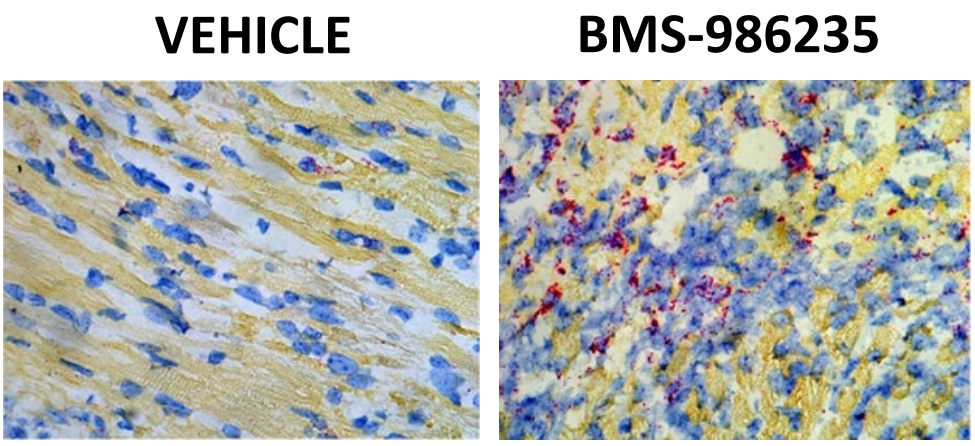


Figure S6.

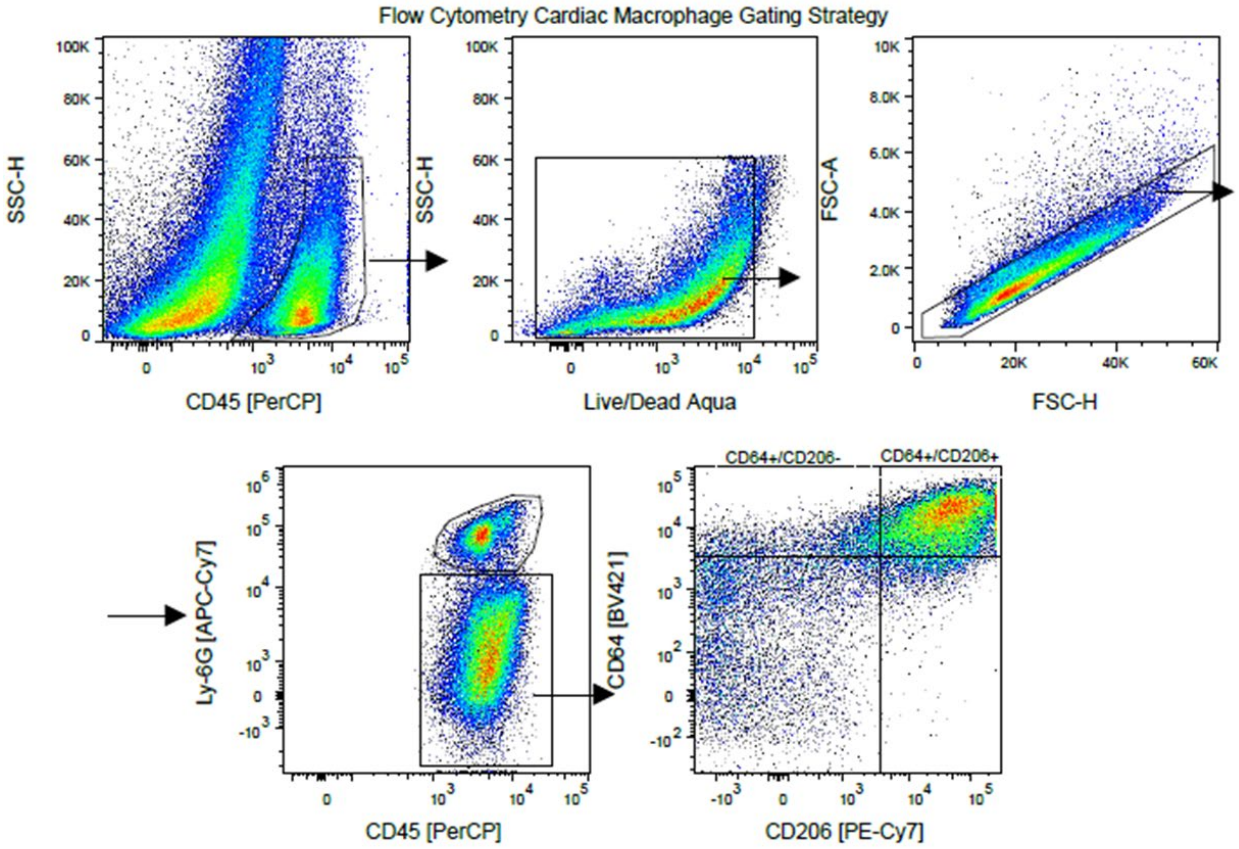

Flow Cytometry Cardiac Macrophage Gating Strategy
SSC-H
CD45 [PerCP]
SSC-H
Live/Dead Aqua
FSC-A
FSC-H
Ly-6G [APC-Cy7]
CD45 [PerCP]
CD64 [BV421]
CD206 [PE-Cy7]
CD64+/CD206-
CD64+/CD206+

Figure S7.

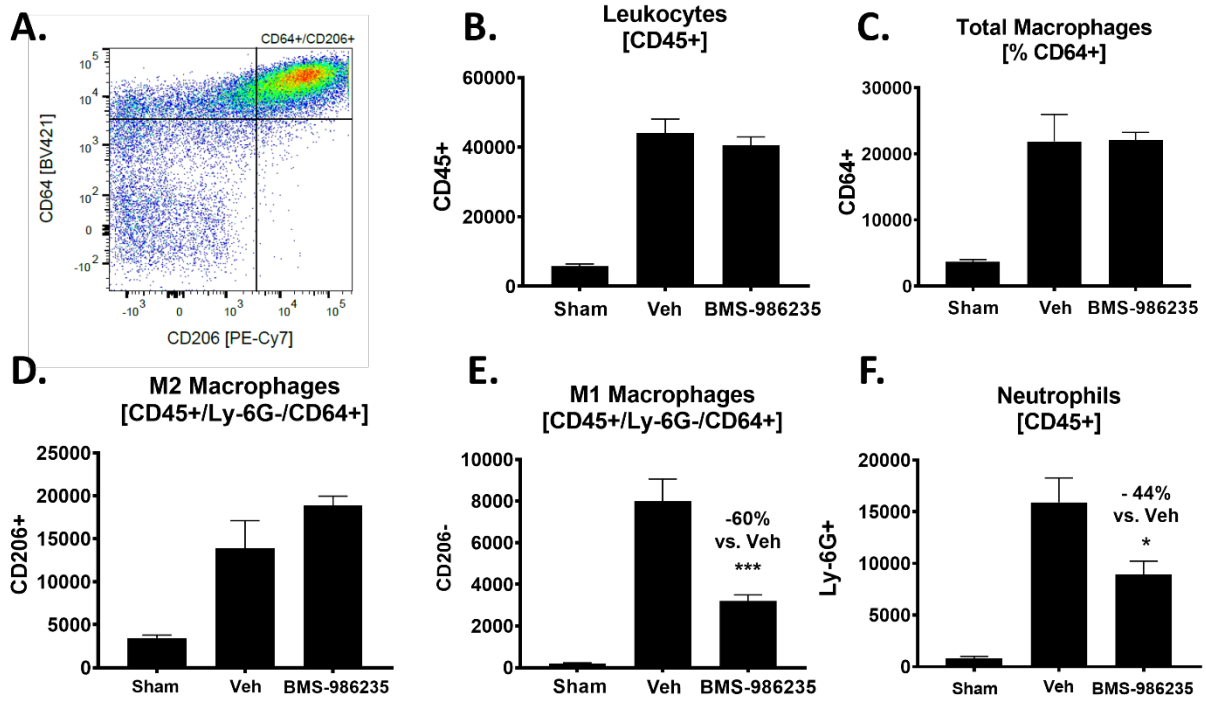

A.
CD64+/CD206+
CD64 [BV421]
CD206 [PE-Cy7]
B.
Leukocytes
[CD45+]
CD45+
Sham
Veh
BMS-986235
C.
Total Macrophages
[% CD64+]
CD64+
Sham
Veh
BMS-986235
D.
M2 Macrophages
[CD45+/Ly-6G-/CD64+]
CD206+
Sham
Veh
BMS-986235
E.
M1 Macrophages
[CD45+/Ly-6G-/CD64+]
CD206-
-60%
vs. Veh
***
Sham
Veh
BMS-986235
F.
Neutrophils
[CD45+]
Ly-6G+
- 44%
vs. Veh
*
Sham
Veh
BMS-986235

Figure S8.

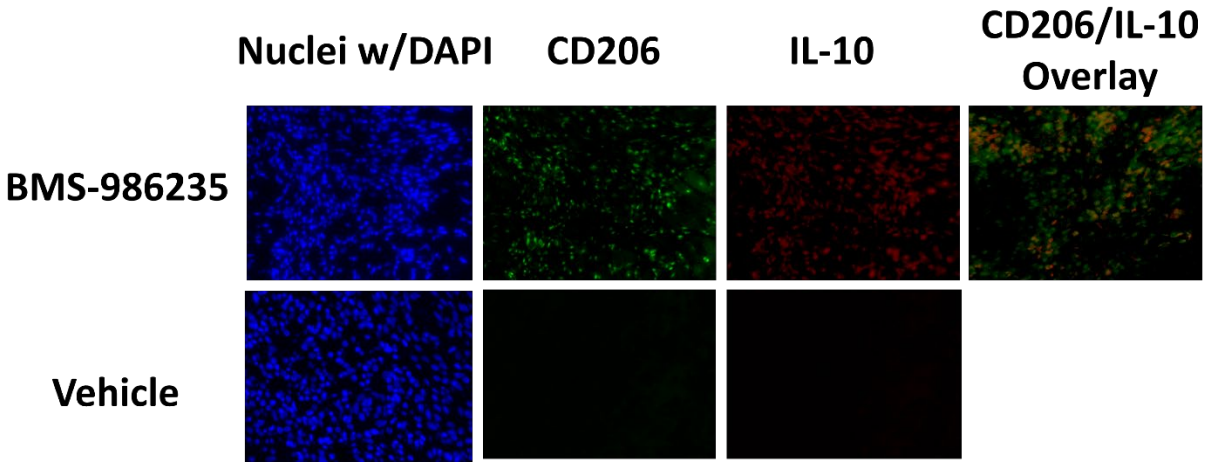

Nuclei w/DAPI
CD206
IL-10
CD206/IL-10 Overlay
BMS-986235
Vehicle

Figure S9.

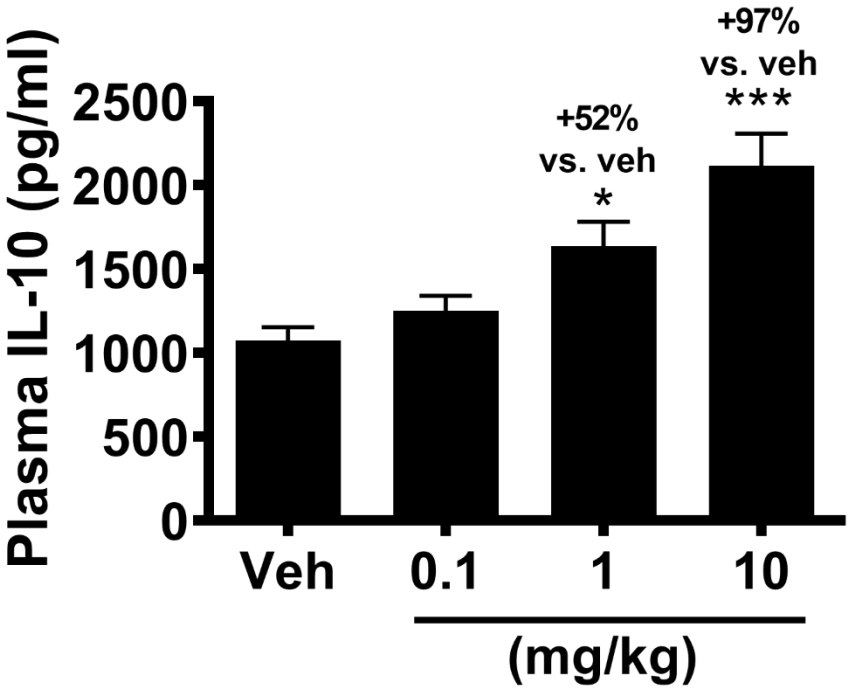

Plasma IL-10 (pg/ml)
2500
2000
1500
1000
500
0
+52%
vs. veh
*
+97%
vs. veh
***
Veh
0.1
1
10
(mg/kg)

Figure S10.

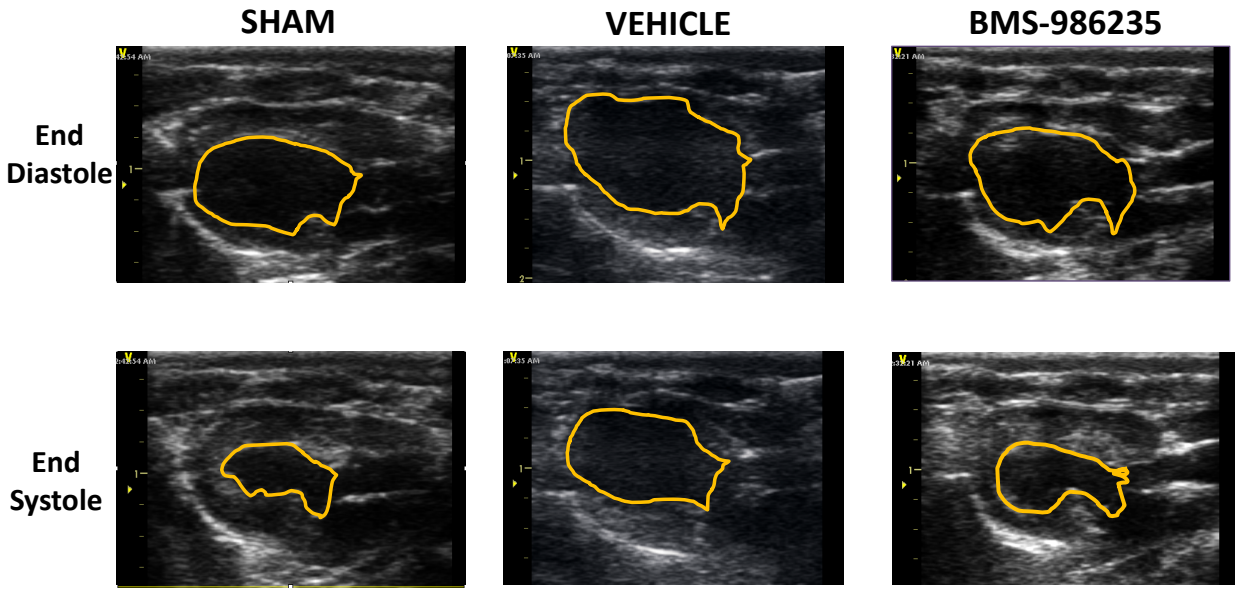


Figure S11.

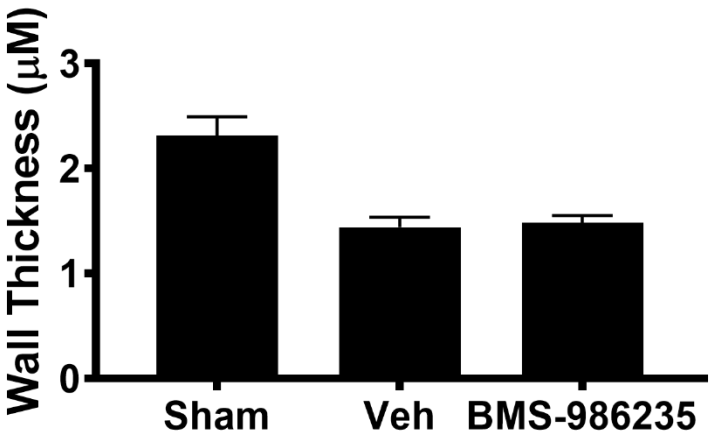
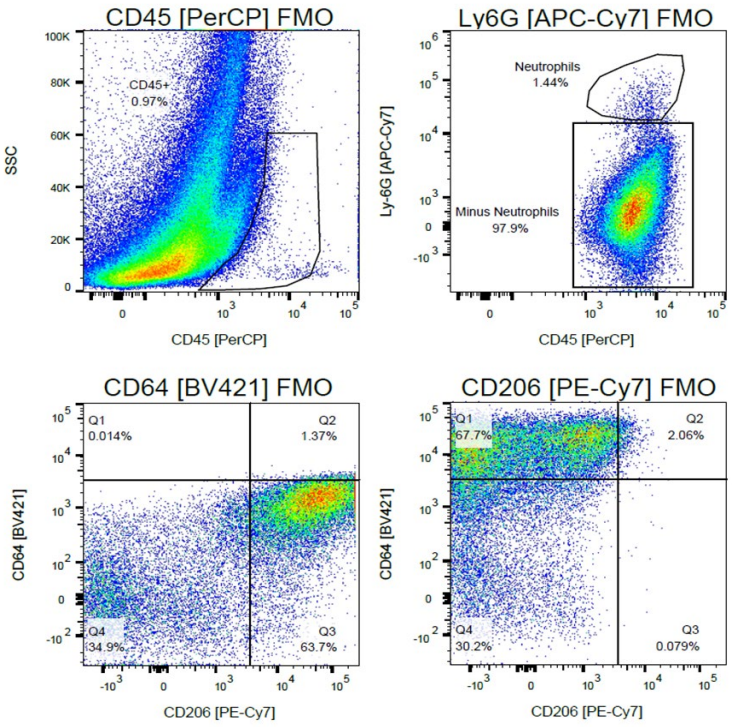


Figure S12.


CD45 [PerCP] FMO
SSC
CD45+
0.97%
CD45 [PerCP]
Ly6G [APC-Cy7] FMO
Neutrophils
1.44%
Minus Neutrophils
97.9%
Ly-6G [APC-Cy7]
CD45 [PerCP]
CD64 [BV421] FMO
Q1
0.014%
Q2
1.37%
Q4
34.9%
Q3
63.7%
CD64 [BV421]
CD206 [PE-Cy7]
CD206 [PE-Cy7] FMO
Q1
67.7%
Q2
2.06%
Q4
30.2%
Q3
0.079%
CD64 [BV421]
CD206 [PE-Cy7]

Supplemental References

1. Asahina Y, Wurtz NR, Arakawa K, et al. Discovery of BMS-986235/LAR-1219: A Potent Formyl Peptide Receptor 2 (FPR2) Selective Agonist for the Prevention of Heart Failure. J. Med. Chem. 2020;63:9003–9019.

2. Folland ED, Parisi AF, Moynihan PF, Jones DR, Feldman CL, Tow DE. Assessment of left ventricular ejection fraction and volumes by real-time, two-dimensional echocardiography. A comparison of cineangiographic and radionuclide techniques. Circulation 1979;60:760–766.

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