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Mast Cells Mediate Early Neutrophil Recruitment and Exhibit Anti-Inflammatory Properties via FPR2/ALX

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ABSTRACT

Background and purpose

In recent years, studies have focused on the resolution of inflammation, which can be achieved by endogenous anti-inflammatory agonists such as Annexin A1 (AnxA1). Here, we investigated the effects of mast cells (MCs) on early lipopolysaccharide (LPS)-induced neutrophil recruitment and the involvement of the AnxA1-Formyl peptide receptor 2/ALX (Fpr2/ALX or lipoxin A_4 receptor) pathway.

Experimental approach

Intravital microscopy (IVM) was used to visualize and quantify the effects of LPS (10 µg per mouse i.p.) on murine mesenteric cellular interactions. Furthermore, the role MCs play in these inflammatory responses was determined *in vivo* and *in vitro* and effects of AnxA1 mimetic peptide Ac2-26 were assessed.

Key Results

LPS increased both neutrophil endothelial cell interactions within the mesenteric microcirculation and MC activation (determined by IVM and ruthenium red dye uptake) which in turn lead to the early stages of neutrophil recruitment. MC recruitment of neutrophils could be blocked by preventing the pro-inflammatory activation (using cromolyn sodium) or enhancing an anti-inflammatory phenotype (using Ac2-26) in MCs. Furthermore, MCs induced neutrophil migration *in vitro* and MC stabilization enhanced the release of AnxA1 from neutrophils. Pharmacological approaches (such as the administration of Fpr pan-antagonist Boc2, or the selective Fpr2/ALX antagonist WRW4) revealed neutrophil Fpr2/ALX to be important in this process.

Conclusions and Implications

Data presented here provides evidence for a role of MCs, which are ideally positioned in close proximity to the vasculature, to act as sentinel cells in neutrophil extravasation and resolution of inflammation via the AnxA1-Fpr2/ALX pathway.

Abbreviations

PPE - Plasma protein extravasation

MCP-1 - Monocyte chemoattractant protein-1

MIP-1α - Macrophage inflammatory protein 1-alpha

KC - Keratinocyte chemoattractant

IL-8 - Interleukin-8

TLRs - Toll-like receptors

TLR-2 - Toll-like receptor-2

ROS - Reactive oxygen species

NETs - Neutrophil extracellular traps

FPRs - Formyl peptide receptors

AnxA1 - Annexin A1

Ac2-26 – Annexin A1 mimetic peptide (Ac-AMVSEFLKQAWFIENEEQEYVQTVK)

H & E - Hematoxylin and eosin

Boc2 - *N-tert*-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe (pan-antagonist of the formyl peptide receptors)

DMSO - Dimethyl sulfoxide

PBS – Phosphate buffered saline

fMLP – formyl-Met-Leu-Phe

WRW4 - FPR2/ALX selective synthetic compound

FITC - Fluorescein isothiocyanate

aN - Adherent neutrophils

eN - Emigrated neutrophils

IL-6 - Interleukin-6

TNF-α - Tumor necrosis factor alpha

IL-1ß - Interleukin 1 beta

MC – Mast Cell

LXA_4 - Lipoxin A4

CMP 48/80 – Compound 48/80

LPS - Lipopolysaccharide

IVM – Intravital microscopy

V_{WBC} - White blood cell velocoty

WSR – Wall shear rate

FL_{in} - Fluorescence intensity inside the vessel

FL_{out} - Fluorescence intensity outside the vessel

Bk - Background fluorescence

MCET - Mast cell extracellular trap

INTRODUCTION

The innate immune system is the first line of defence against invading pathogens such as fungi or bacteria. Within the heterogenic cell populations of the innate immune system, neutrophils are the most abundant leukocyte population (50–70%) in human blood, and as such are well recognized as a major player during acute inflammation, being quickly directed to sites of infection/injury (Nathan, 2006). Upon activation, neutrophils engulf bacteria or release a variety of factors, including reactive oxygen species (ROS), and facilitate the formation of neutrophil extracellular traps (NETs) to capture and destroy pathogens (Serhan *et al*., 2008; Segal, 2005; Yipp *et al*., 2013). Since neutrophil activation can mediate tissue injury and perpetuate the inflammatory response their physiological effector functions are tightly mediated and regulated via cell-surface receptors (e.g. toll like receptors (TLRs)) (Segal, 2005; Chiang *et al*., 2006).

Resolution of inflammation normally occurs when the invading pathogen has been neutralized and before the immune response becomes pathological. However, failure to resolve inflammation can lead to excessive or deregulated neutrophil responses, which (together with inadequate repair) contribute to the persisting tissue damage that underlies many infectious, ischemic or inflammatory diseases, e.g. stroke, sepsis and myocardial infarction (Nathan, 2006; Borregaard, 2010). It is now appreciated that the host response to inflammatory insults involves tightly controlled and active (rather than passive) resolution programs (Chiang *et al*., 2006; Serhan *et al*., 2008), in which efficient resolution depends on inhibition of neutrophil influx, rapid clearance of infiltrating neutrophils and regeneration of disrupted tissue structures (Serhan *et al*., 2007).

Neutrophils undergo a sequential pattern of interaction with vascular endothelial cells, orchestrated by a well-characterized sequence of rolling, adhesion and emigration into inflamed/infected tissue (Hughes *et*

al., 2013). Mast cells (MCs) are an important source of many pro-inflammatory mediators, including those that activate the expression and/or activation of adhesion molecules involved in the leukocyte recruitment cascade (Kubes *et al*., 1996, Yazid *et al*., 2010). In particular the close proximity of MC to microcirculatory vessels puts them in a prime position to activate and direct circulating neutrophils. In addition, MCs are rather promiscuous and also appear to play a protective role in regulation of innate immune responses. For example, MCs have been shown to aid the resolution of inflammation by mobilizing neutrophils to the site of infection and by producing endogenous anti-inflammatory and pro-resolving mediators such as annexin A1 (AnxA1) (da Silva *et al*., 2011).

The 37 kDa protein AnxA1 (and its mimetic peptide Ac2-26) is a potent inhibitor of leukocyte infiltration. It regulates leukocyte detachment from the postcapillary endothelium in both acute and chronic inflammatory states (Gavins *et al*., 2012), and deletion of the *AnxA1* gene is associated with exacerbated inflammatory responses (Hannon *et al*., 2003). AnxA1 is widely distributed, being detected in e.g. lung, kidney, bone marrow, intestine, spleen, thymus or brain (Shantel *et* al., 2016; Gavins *et al*., 2012; Fava *et al*., 1989), and secreted by a variety of cell types including MCs (Kwon *et al*., 2012) and neutrophils (Oliani *et al*., 2001). AnxA1's effects are mediated through a common G protein-coupled receptor family: the formyl peptide receptors (FPRs). FPR2/ALX (or LXA_4 receptor, termed Fpr2/3 in the mouse) (Shantel *et al*., 2016) is expressed on endothelial cells and cells of myeloid lineages (Chiang *et al*., 2006) and is strongly associated with the anti-inflammatory effects of AnxA1 during resolution (Shantel *et al*., 2016; Serhan *et al*., 2008).

We and others have shown that AnxA1 plays a significant role in limiting inflammatory responses (Shantel *et al*., 2016, Hughes *et al*., 2013), and that stimulation of phosphorylation and subsequent

release of AnxA1 is an important component of the inhibitory actions of MC-stabilizing drugs (Yazid *et al*., 2011; Yazid *et al*., 2013). However, the effect of MCs on early neutrophil recruitment and the role of the AnxA1-Fpr2 pathway has not been extensively investigated. We used IVM, coupled with pharmacological approaches to investigate the interaction of MCs and the AnxA1-Fpr2 /ALX pathway in early neutrophil recruitment.

METHODS

Animals

Male mice aged 5–8 weeks were used and maintained on a 12 h light-dark cycle with room temperature at 21–23°C and access to a standard chow pellet diet and tap water *ad libitum*. C57BL/6 mice were purchased from either Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Limited (Margate, Kent, UK). All animal experiments complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and followed the European Union Directive (2010/63/EU) or were approved by the Louisiana State University Health Sciences Center Shreveport Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

Drugs

The following drugs were used and dissolved in sterile saline unless otherwise stated: Zymosan A (Sigma-Aldrich, Poole, Dorset, UK), ammonium thioglycollate (thioglycollate, Sigma, UK), cromolyn sodium (Sigma, UK), AnxA1 mimetic peptide Ac2-26 ($AnxA1_{Ac2–26}$, Ac-AMVSEFLKQAWFIENEEQEYVQTVK, Cambridge Research Biochemicals, Cleveland, UK. dissolved in phosphate buffered saline (PBS)), compound 48/80 (CMP 48/80, Sigma, UK), Boc2 (*N*-*tert*-butoxycarbonyl-L-Phe-D-Leu-L-Phe-D-Leu-L-Phe; MPBiomedicals, Cambridge, UK. Dissolved in 0.02% dimethyl sufoxide (DMSO) and saline), WRW4 (Trp-Arg-Trp-Trp-Trp-NH_2, Tocris, Bristol, UK). Fluorescein isothiocyanate (FITC)-labelled albumin (0.25 mg/g body weight, Sigma, UK) was administered intravenously (i.v.) to enable visualization of plasma leakage.

Induction of experimental endotoxaemia

Endotoxaemia was induced by intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, endotoxin, *E. coli* serotype 0111:B4, 10 µg/mouse, Sigma, UK) while controls received a corresponding volume (50 µl, i.p.) of saline vehicle. We chose to use a low dose of LPS within the study in order to cause activation of the vasculature but not provoke decreased microvascular perfusion or mortality which occurs with higher LPS doses and would complicate interpretation of the inflammatory response (Zanetti *et al*., 1992; Damazo *et al*., 2005). To ascertain the time-course of the responses to LPS, mesenteric leukocyte activity was visualized by IVM 0, 10, 20, 60 or 120 min after injection. In addition serum pro-inflammatory cytokines TNF-α, IL-1ß and IL-6 were also determined 0, 10, 20, 60 or 120 min after injection by ELISA as per manufactures (R & D Systems, Minneapolis, MN). To ascertain the specificity of the response to LPS, zymosan (500 µg/mouse, i.p.) and thioglycollate (250 µl of a 3% solution, i.p.) were injected in place of LPS. In pre-treatment experiments mice received sodium cromolyn (10 mg/kg, i.p.), Ac2-26 (100 µg/mouse, i.v.) or saline vehicle 15 min prior to LPS injection.

Visualization of the mesentery by IVM

Anaesthesia was induced with an i.p. injection of ketamine (Ketaset, 150 mg/kg, Fort Dodge Animal Health, Southampton, UK) and xylazine (Rompun, 7.5 mg/kg, Bayer Healthcare, Newbury, UK) and topped up as required (upon presence of a pedal reflex). The jugular vein was exposed and cannulated with polyethylene tubing (PE10) for drug administration. A midline incision was made, the mesenteric vascular bed was exteriorized, gently laid across a Plexiglass viewing stage, and mounted on an Olympus BW61WI microscope with a water-immersion objective lens (magnification of 40x, LUMPlan, FI/IR, Olympus, Japan). The tissue preparation was transilluminated with a 12-V, 100 W halogen light source. The mesenteric vessels were superfused with bicarbonate-buffered solution (g/L: NaCl, 7.71; KCl, 0.25; MgSO4, 0.14; NaHCO3, 1.51; and CaCl2, 0.22, pH 7.4) and 1–5 randomly selected post-

capillary venules (20–40 μm wide and 100 μm long) were observed per mouse. Leukocyte rolling velocity (V_{WBC}) was measured and calculated in μm/s. Leukocyte adhesion was measured by counting clearly visible cells that remained stationary on the vessel wall for at least 30 s, within the 100 μm stretch. Leukocyte emigration from the microcirculation into the tissue was quantified by counting the number of cells 50 μm outside the vessel wall on either side of the 100 μm stretch. Real-time videos of the vessels were made with a black-and-white camera (model CoolSNAP HQ^2, Photometrics, Tucson, AZ) coupled to a Windows XP-based computer for recording by Slidebook 4.2 (Intelligent Imaging Innovations, Inc., Denver, CO).

Tissue processing and staining

Mice were anaesthetised using ketamine (150 mg/kg) and xylazine (7.5 mg/kg) i.p. for transcardial perfusion with 10 ml PBS followed by 10 ml formaldehyde. Tissues were fixed in formaldehyde solution overnight before routine processing to paraffin wax. 4 μm sections were cut on a microtome and stained with haematoxylin and eosin.

Plasma protein extravasation

FITC-labelled albumin was injected i.v. (0.25 mg/gram body weight) 5–10 min prior to the end of the experiment. A snapshot of vessel fluorescence was taken using block filter (excitation at 450–490 nm, emission at 535–620 nm). Albumin leakage was quantified by measuring mean fluorescence intensity using ImageJ64 (National Institute of Health, USA). Average fluorescence intensity in three areas of equal size was measured: inside the vessel (Fl_{in}), outside the vessel (Fl_{out}) and background fluorescence in an area with no obvious leakage (bk). Albumin leakage was then determined as: $[(Fl_{out} \times bk)/(Fl_{in} \times bk)] \times 100\ \%$.

MC visualization and activation

Mice were treated with LPS or saline vehicle and after 20 min, the mesentery was exteriorised as described above and superfused with bicarbonate buffered saline containing 0.001% ruthenium red, which is selectively taken up by activated MCs (da Silva *et al*., 2011). Images of the mesenteric vascular bed were recorded under a X10 objective every 5 min over a 15 min period, maintaining the same field of view. After that time the MC degranulating agent CMP 48/80 (1 μg/ml) was added to the superfusion buffer to cause maximum activation of the MCs and a series of final snapshots was taken after 1, 5 and 10 min. MC activation was analysed using ImageJ64, where images were converted to black and white and single MCs were selected for analysis. The freehand tool was used to measure the greyscale intensity at each timepoint, expressed as a percentage of the maximum (taken as the final snapshot 10 min after CMP48/80 superfusion). 3–5 MCs were analysed per field of view and calculated as the mean.

MC isolation

Peritoneal leukocytes (2×10^8 cells/ml) were fractionated on a preformed continuous gradient generated from 70% isotonic Percoll in 0.15M NaCl (Amersham Pharmacia Biotech, NJ, USA). The gradient was calibrated between 1.018 and 1.138 g/mL with density marker beads (Amersham Biosciences). MCs were collected at band density of 1.088 g/mL and their purity was >95% positive when tested by flow cytometry using CD117–fluorescein isothiocyanate mAb (BD Biosciences, Oxford, England).

MC stimulation

Purified peritoneal MC at 0.5×10^5 cells/ml were stimulated in 24-well plates (VWR, Leicestershire, England) with saline or LPS (1 μg/ml) for 4 h or LPS (1 μg/ml) and CMP 48/80 (10 μg/ml) for 20 min at

37°C with 5% CO_2 atmosphere, with or without cromolyn sodium. The cell-culture supernatants were either removed, spun, and analyzed by enzyme-linked immunosorbent assay (ELISA) for the presence of histamine (SPI bio, Strasbourg, France) or AnxA1 (MyBiosource Inc, San Diego, USA), or were added to chemotaxis plates.

Neutrophil isolation

Mouse bone marrow cells were harvested by flushing marrow from femurs and tibias with RPMI-1640 medium. Neutrophils were isolated from bone marrow cells by density centrifugation with Histopaque-1077 and Histopaque-1119. The purity of isolated neutrophils was routinely > 95% as assessed by light microscopic analysis of the cells stained with Diff-Quick (Wako Pure Chemical Industries, Osaka, Japan), and > 98% viable as assessed by a trypan blue exclusion test.

Neutrophil labelling

Isolated neutrophils were stained with Calcein AM (Sigma, UK; 1µl of Calcein per 4 million cells) for 30 min at 37 °C with 5% CO_2 atmosphere. The cells were then washed and used in the chemotaxis assay.

Neutrophil chemotaxis assay

MCs were degranulated as described above and the supernatant was collected. Neutrophils (4 x 10^6 cells/ml) were added to the upper chamber of a Neuroprobe 96-well disposable chemotaxis plate (Neuro Probe Inc. MD, USA, 5 µm pore size) in RPMI containing 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. MC supernatant was added to the lower chamber. Plates were incubated for 1.5h (37.5°C, 5% CO_2). The number of migrated cells were assessed

using a spectrofluorometer (excitation 485 nm and emission 528 nm). In some cases calcein-AM labelled neutrophils were pre-incubated (10 min) with Boc2 (20 μM) or WRW4 (10 μM) prior to being incubated with the MC supernatant described above. This neutrophil-MC supernatant cocktail was added to the upper chamber of a Neuroprobe chemotaxis plate and vehicle or the chemoattractant fMLP (10 nM) added to the lower wells. Plates were incubated for 1.5 h (37.5°C, 5% CO_2) and read as above.

Myeloperoxidase (MPO) activity

Mesenteric tissue samples were collected from mice at 0, 20, 60 or 120 min post LPS or saline vehicle treatment. MPO was measured as a marker for mesenteric neutrophil infiltration. Mesenteric tissue homogenates and MPO standards (Sigma, UK) were placed onto a 96-well plate, and 200 μL of *o*-dianisidine (Sigma, UK) solution and 10 μL of 0.1 % H_2O_2 (Sigma, UK) were added. The absorbance was read after 5 min at 405 nm and expressed as units per mg of wet tissue (Gavins *et al*., 2007).

Histamine release

Histamine release was measured using an ELISA (SPI Bio). The assay was conducted following the manufacturer's protocols. A standard curve ranging from 0.39–50 nM histamine was prepared using the reagent provided and the optical density was then read within 60 min in a microplate reader (Titertek™, Vienna, Austria) at 405 nm.

AnxA1 ELISA

AnxA1 release was measured using an ELISA (MyBiosource). The assay was conducted following the manufacturer's protocols. A standard curve ranging from 0.625–40 ng/ml was prepared using the

reagent provided and the optical density was then read within 60 min in a microplate reader (Titertek™) at 450 nm.

Data analysis and statistical procedures

All data were analyzed using GraphPad Prism 6. Data are expressed as mean ± SEM with n values given in the respective figure legends. When determining statistical significance between two groups an unpaired *t*-test was carried out and where appropriate, corrected for multiple comparisons using the Holm-Šídák method. Multiple groups were analyzed by one-way ANOVA or non-parametric Kruskal–Wallis test and *post-hoc* comparisons were performed by Bonferroni or Dunn's multiple comparison test respectively. Differences were considered statistically significant if $p < 0.05$. The figures have been graphically presented on a range-specific axis. All data and statistical analysis comply with British Journal of Pharmacology guidelines (Curtis *et al.*, 2015).

RESULTS

LPS induces early leukocyte-endothelial cell interactions in the murine mesentery

LPS induced heightened cellular trafficking within the mesenteric microcirculation as early as 20 min when determined by IVM. These findings are displayed in the representative image of the mesenteric vascular bed taken 20 min post LPS challenge (Figure 1A). Whilst no increase in leukocyte adhesion at that early time-point was observed (Figure 1C), the number of emigrated leukocytes had already increased significantly (saline = 2.4 ± 0.3 cells vs. LPS = 5.8 ± 0.6 cells; Figure 1D). Both parameters (Figure 1C + D), as well as leukocyte rolling velocity (Figure 1B) and wall shear rate (Table 1), were significantly ($p < 0.001$) higher in LPS treated mice at 60 and 120 min.

LPS induces early mesenteric but not systemic inflammatory responses

Another cardinal sign of an inflammatory response, plasma protein extravasation (PPE) measured by FITC-albumin leakage using IVM, was found to follow the same pattern as leukocyte emigration: LPS induced an acute increase at 20 min post treatment, which remained significantly elevated at 60 and 120 min (Figure 1 E + F. $p < 0.001$). Despite the initial acute local inflammatory responses in the mesentery, no early systemic effects were recorded when the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were measured in serum of saline- or LPS-treated mice (Table 2). All three cytokines were found to be significantly ($p < 0.001$) raised at 60 and 120 min following LPS injection.

The early acute inflammatory response is not specific to LPS

Having established that an early cellular recruitment (i.e. emigration of leukocytes) occurs in the murine mesenteric microvasculature in response to LPS, we next tested whether this phenomenon was pathogen-specific. We used two additional inflammagens: zymosan, a glucan derived from yeast acting

as a ligand for toll like receptor-2 (TLR-2) (Volman *et al*., 2005) and thioglycollate, a non-specific leukocyte chemotactic agent (Vemula *et al*., 2010). When comparing rolling velocity, we found both zymosan and thioglycollate to induce a significant increase when compared to saline control as well as to LPS (Figure 2A) whilst only thioglycollate was found to increase leukocyte adherence at 20 min. (Figure 2 B). In regards to leukocyte emigration, zymosan followed the established trend for LPS and caused increased extravasation, whilst thioglycollate did not differ from saline control (Figure 2C). Finally, both inflammagens mirrored the effects observed with LPS on PPE (Figure 2D). Although the experiments showed that the acute inflammatory mesenteric response was not specific to LPS, we continued to use LPS throughout the rest of the study based on the superior inflammatory profile in regards to endothelial-leukocyte interactions.

Neutrophils are the predominant cell type in the inflamed murine mesentery

Since Hogg *et al*., previously described the inflammatory response to LPS in the murine mesentery to be mainly mediated by neutrophils, we aimed to confirm these results in our model. Analysis of histological slides showed the above mentioned leukocyte population to consist predominantly of neutrophils (Figure 3A). Having this established, we used a second neutrophil assay: MPO, as a marker of neutrophil infiltration and found a significant increase within the mesentery as early as 20 min after LPS treatment (Figure 3B). These results first confirm the observations made by IVM of an early LPS induced leukocyte emigration in the murine mesentery and second identifies these cells to be predominantly neutrophils.

LPS causes an early MC activation in the mesentery

As MCs lie in close proximity to vessel walls, we hypothesized that the early LPS-induced inflammatory response in the mesenteric microvasculature could be at least in part driven by LPS-induced MC activation. Therefore, we tested the influence of LPS on early MC activation in the murine mesentery and as such, MC activation was evaluated by measuring the uptake of ruthenium red dye (expressed at each timepoint as % of the maximum uptake after complete stimulation with CMP 48/80), which is selectively and quantitatively taken up by activated MCs. We found a significantly increased uptake of dye in the LPS group, observable within 5 min of treatment (Figure 4A). To further determine the degree of early MC activation following LPS stimulation we used the established MC activator CMP 48/80 to test if this would result in an additional MC activation above LPS-induced levels. Importantly, in saline-treated mice MCs were rapidly activated upon addition of CMP 48/80, whilst those from LPS-treated mice did not further respond to that stimulus suggesting a near-complete early MC activation following LPS treatment. (Figure 4A).

MC activation is involved in the early inflammatory response in the mesenteric microvasculature

Having established MC to be activated early by LPS, we next tested the hypothesis that MC activation was dynamically involved in the acute inflammatory responses (neutrophil adherence and emigration as well as PPE) being observed in the murine mesenteric microvasculature. Thus, we first administered the non-specific MC stabilizer cromolyn sodium, to prevent MC activation towards a pro-inflammatory phenotype. In addition, as AnxA1 has previously been described to be a key regulator of MC reactivity, acting as an endogenous MC stabilizer and to further promote the release of anti-inflammatory mediators, we further aimed to test the influence of the AnxA1 mimetic peptide Ac2-26. When analyzing leukocyte-endothelial cell interactions and PPE 20 min after LPS stimulation, we found that

neither pre-treatment with cromolyn sodium nor Ac2-26 had any significant influence on the previously described slight differences in cell adherence (Figure 4B). However, both reagents significantly reduced the numbers of emigrated neutrophils ($p < 0.01$ respectively, Figure 4C) as well as PPE ($p < 0.01$ respectively, Figure 4D). These results demonstrate that both preventing the pro-inflammatory activation (cromolyn sodium) as well as enhancing an anti-inflammatory phenotype (Ac2-26) in MCs, were able to regulate the early *in vivo* inflammatory response in the mesenteric microvasculature.

MCs are a source of AnxA1 and mediate early neutrophil recruitment via AnxA1-Fpr2/ALX

Having established that MC activation is involved in early neutrophil migration and PPE, and that Ac2-26 (enhancing the anti-inflammatory phenotype in MCs) is able to block this process like cromoyln sodium *in vivo*, we aimed to further investigate the relationship of neutrophils and MCs via the AnxA1-Fpr2/ALX pathway in the sequence of neutrophil recruitment *in vitro*. Using an *in vitro* transmigration assay, equiped with freshly isolated MC from the peritoneal cavity (Filippo *et al*., 2014) and bone marrow derived neutrophils, we found that supernatant collected from the LPS-stimulated MC had chemotactic activity and was able to promote neutrophil migration *in vitro* (Figure 5A). Furthermore, the LPS-stimulated MC supernatant induced an increase in AnxA1 release from neutrophils (as measured by ELISA, Figure 5B).

Next we took purified MC and pre-treated them with cromolyn or saline followed by a further (20 min) stimulation with either saline or LPS + CMP48/80. Using the obtained supernatant, we then tested it for presence of the key MC-derived pro-inflammatory molecule, histamine. LPS + CMP48/80 treatment resulted in a significant increase in histamine release (Figure 5C), which was blocked by the pre-treatment with cromolyn. Since previous studies have shown that cromones are able to provoke the

release of AnxA1 from cord-derived human MCs (Yazid *et al*., 2010), we next quantified the release of AnxA1 from cells in the above described set of experiments. We found that LPS + CMP48/80 resulted in a significant release of MC-elicited AnxA1, which was exacerbated when cells were pre-treated with the MC stabilizer cromolyn, enhancing the MCs' anti-inflammatory phenotype.

Finally, having demonstrated that MC not only promote the release of AnxA1 from neutrophils, but when stabilized present an anti-inflammatory phenotype (demonstrated by decreased production of histamine and increase in AnxA1 release), we wanted to address whether, when MC and neutrophils are coupled together, the production of AnxA1 from MC acts on neutrophils to help resolve inflammation. Figure 5E shows that the combination of neutrophils coupled with the supernatant from stimulated (LPS + CMP48/80) MC, results in a robust *in vitro* neutrophil migration torwards the chemoattractant fMLP. This response was significantly reduced when MC were pre-treated with cromolyn. Based on our results in Figure 4 D, this suggests a role for AnxA1 in limiting neutrophil recruitment and therefore we tested this hypothesis by pre-incubating neutrophils with either the FPR pan-antagonist Boc2 or the FPR2 selective antagonist WRW4 before exposure to MC supernatant. Both Boc2 and WRW4 caused a significant increase in neutrophil migration vs. vehicle, suggesting the importance of neutrophil Fpr2 in this process of MC-mediated neutrophil migration via AnxA1.

DISCUSSION

We have used the technique of IVM, coupled with pharmacological approaches, to assess hemodynamic parameters within the mesenteric microcirculation, and the state of MC activation surrounding the vascular bed under study. We have demonstrated that upon LPS stimulation, MCs contribute to the early stages of neutrophil recruitment and that this process could be blocked by preventing the pro-inflammatory activation (using cromolyn sodium) or enhancing an anti-inflammatory phenotype (using Ac2-26) in MCs. We further demonstrated *in vitro* that stimulated MCs induce neutrophil migration and that MC stabilisation enhances the release of AnxA1, which acts upon neutrophil Fpr2/ALX to limit further neutrophil recruitment. These findings demonstrate thus a positive role for MCs in the resolution of inflammation.

MCs are multifunctional and highly effective tissue-dwelling cells found in most tissues of the body, especially in sites that are in close contact to the external environment e.g. skin, airways, and intestines. Within seconds of stimulation, MC become activated, degranulate and release a variety of soluble factors including: histamine, proteases e.g. tryptase and chymase, and pre-formed TNF-α, followed by the production of lipid-derived eicosanoids e.g. prostaglandin D_2 and leukotriene C_4 (Urb *et al*., 2012). MCs are also capable of selectively releasing pro-inflammatory mediators, such as IL-6, without degranulation (Theoharides *et al*., 2012). In the present study, exposure of the mouse mesentery to CMP48/80 (which bypasses the high-affinity IgE receptor and acts directly on G-proteins to produce MC degranulation) causeda rapid selective uptake of ruthenium red uptake by MCs, demonstrating that MCs are activated very rapidly after stimulation (Urb *et al*., 2012). Furthermore, it suggests that MCs were playing a role in the early neutrophil recruitment and oedema formation.

Whilst MCs clearly participate in the induction and/or propagation of certain inflammatory diseases (Theoharides *et al*., 2012) and have been best characterized by their role in allergic reactions, there is growing interest in understanding how MCs may play a role in immunoregulation, in leukocyte recruitment (Abraham and John, 2010; de Filippo *et al*., 2013). Depletion of MCs causesheightened acute neutrophil emigration and oedema, suggesting that MCs play a protective role in terms of neutrophil emigration and albumin leakage in response to LPS. Futher evidence for a protective role of MCs has emerged from studies involving MC deficient mice. For example, Echtenacher *et al*., (1996) showed that $Kit^{W}/Kit^{W\text{-}v}$ (MC-deficient) mice display a significantly increased mortality rate versus their wild-type counterparts following acute septic peritonitis, which could be corrected by restoration of the deficiency. Thus, MCs are required for host defence in some murine models of bacterial infection, although the mechanisms utilized have until now been largely undefined.

Previous studies have shown that MCs do not degranulate *in vitro* in response to LPS (Theoharides *et al*., 2000), however acting via TLR4, LPS is able to cause the secretion of a number of different factors from murine MCs including: IL-6, IL-13, and TNFα (McCurdy *et al*., 2001; Supahatura *et al*., 2001). Our data concur with findings of other groups (di Filippo *et al.,* 2013) that LPS-stimulated MC supernatant has chemotactic activity. Furthermore, there was an increase in AnxA1 release from MCs into the supernatant. AnxA1 is present in a number of myeloid cells including neutrophils and MCs (Oliani *et al*., 2000) and once secreted from existing intracellular pools of activated cells, acts in a paracrine/autocrine fashion, utilising FPRs to produce its biological effects (Yazid *et al*., 2010; Perretti M and D'Aquisto. 2009).

AnxA1 is an endogenous regulator of MC function, and administration of this protein has been shown to exert a tonic inhibitory influence on MC reactivity e.g. administration of Ac2-26 to rats inhibits antigen-induced histamine release in the pleural cavity (Bandeira-Melo *et al*., 2005). AnxA1 is cleaved in activated MCs and this cleavage is required for the phosphorylation of cytosolic phospholipase A_2 and subsequent inflammatory reactions, such as the production of eicosanoids.

Cromoglycate-like drugs, e.g. cromoyln sodium, have been described as 'MC stabilizers', due to their ability to prevent the release of histamine when stimulated by different agonists (Yazid *et al*., 2010. It has been confirmed that protein kinase C (PKC) activation is responsible for the phosphorylation of AnxA1 in both cord-derived MCs (Yazid *et al*., 2013) and in U937 cells (Yazid *et al*., 2009), occurring within 5 minutes when stimulated by cromones. Yazid *et al*., also demonstrated that AnxA1 phosphorylation acts either wholly or partially through the FPR system to regulate the limit and extent of mediator release (Yazid *et al*., 2010). It is also important to note that the AnxA1-FPR system can be activated in the absence of MC degranulation by drugs such as glucocorticoids or cromones (Yazid *et al*., 2016). Other groups have also suggested that the G-protein coupled receptor GP35 (which is present on MCs, eosinophils and basophils) also may play a role in the effects of cromones, although this may be more relevant to asthma and allergy rather than infection (Jenkins *et al*., 2010; Yang *et al*., 2010).

The addition of cromoyln sodium in this study resulted in a decrease in neutrophil extravasation and edema. These effects were also mirrored by the administration of Ac2-26. Both these compounds have been shown to inhibit leukocyte emigration e.g. cromoglycate exhibits a protective role in neutrophil-dependent pathologies including intestinal (Kanwar *et* al., 1994) and pulmonary (Vural *et* al., 2000) ischemia-reperfusion models, and AnxA1 and its mimetic peptide have been shown to cause the

detachment of leukocytes in the brain (Gavins *et al*., 2007) and periphery (Gavins *et al*., 2012). Da Silva *et al*., (2011) have also shown the importance of MCs in a model of endotoxin-induced uveitis and suggest that AnxA1 may be an innovative form of therapy for uveitis by preventing MC activation and/or leukocyte infiltration.

The ability of neutrophils to prevent further leukocyte extravasation is clearly complex. Tissue resident macrophages have been previously studied and shown to interact with MCs. De Filippo *et al*., have shown that in response to LPS, MCs are able to quickly recruit neutrophils via release of preformed CXC chemokines (CXCL1/CXCL2), and this is followed by slower macrophage-mediated recruitment of leukocytes deeper into the extravascular tissue (De Filippo et al., 2013). Furthermore, Wolf *et al*., demonstrated that extravasating neutrophils phagocytosed by perivascular macrophages are able to negatively regulate further leukocyte extravasation by secreting a variety of resolution mediators such as resolvins, lipoxins, TGF-β, and IL-10 (Wolf *et al*., 2008). Therefore, it is clear from our study and others that MCs play important modulatory roles in inflammation. Further investigations are required to fully elucidate the functions of these versatile sentinel cells in leukocyte trafficking (Nourshargh and Alon, 2014). Additionally, it must be mentioned that MCs are also able to produce extracellular traps (MCETs, which are composed of DNA, histones, tryptase, and the antimicrobial peptide LL-37) that encompass and kill organisms (von Kockritz-Blickwede *et al*., 2008). However, the importance of these MCETs in clearing all type of inflammation has yet to be established, and it may be that more indirect effects of MCs in coordinating host innate and adaptive responses may be more important in the balance of host defense (von Kockritz-Blickwede *et al*., 2008).

We have demonstrated in this study that MCs play a protective role in the regulation of extravasated neutrophils by the release of endogenous AnxA1, which then acts on neutrophil Fpr2/ALX. MCs also causes the release of AnxA1 from the neutrophil itself, which can act in autocrine and paracrine way to resolve the inflammation. We suggest, based on other data in the literature, that that the feedback of AnxA1 on the MC is likely to be in part dependent upon Fpr1 (Sinniah *et al*., 2016), and not solely on Fpr2/ALX, as we found with the neutrophil. Sinniah *et al*. showed that whilst blocking Fpr2/ALX in cord-derived MCs either pharmacologically (using an antagonist) or genetically (by using cord-derived MCs obtained from Fpr2/3-null mice) prevented the action of nedocromil (MC stabilizing drug) on PGD_2 generation and release, it failed to completely block inhibitory action on histamine release, suggesting the involvement of another FPR (Sinniah *et al*., 2016). These apparent differences in different members of the FPR family mediating the effects of AnxA1 are not uncommon. Intact AnxA1 is a 37-kDa protein, however upon cell activation, AnxA1 can be cleaved at the N-terminal region by proteases such as elastase and proteinase-3 (Vago *et al*., 2016). Differences in signalling could be due the result of a combination of signalling effects produced by AnxA1 or clipped forms e.g. Ac2-26.

In summary, it is important to understand how neutrophils are recruited to control infection or injury. This study provides evidence for a role of MCs as sentinel cells in neutrophil extravasation and resolution of inflammation. Further work is still needed to clarify whether MCs act as first alarm signals to recruit neutrophils upon detection of pathogenic challenge, or whether they are becoming activated by upstream signaling events (Filippo *et al*., 2013). However, this study shows a role for MCs in the resolution of inflammation via the AnxA1-Fpr2/ALX pathway.

AUTHOR CONTRIBUTIONS

ELH designed and performed experiments, interpreted results and helped write manuscript. FB provided scientific input, interpreted results and helped write manuscript. JB designed experiments, provided scientific input and helped write manuscript. RJF provided scientific input and helped write manuscript. FNEG designed and performed experiments, provided scientific input and interpreted results and helped write manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURES AND FIGURE LEGENDS

Figure 1. Effects of LPS on the mesenteric microcirculation. Mice were treated with LPS (10 µg/mouse, i.p.) or saline vehicle. At 0, 20, 60 or 120 min post LPS, the mesentery was exteriorised for visualisation of post-capillary venules by IVM. A) image of mesenteric microcirculation taken 20 min post LPS treatment. Arrows with short tails = rolling leukocytes, arrowheads = adherent leukocytes, arrows with long tails = emigrated leukocytes. Scale bar = 20 µm. Leukocyte-endothelial cell interactions were quantified in terms of B) leukocyte rolling velocity (expressed as V_{WBC}), C) number of adherent (stationary for ≥30 s) leukocytes per 100 µm length and D) number of emigrated leukocytes per 100 x 50 μm^2. FITC-conjugated albumin was injected i.v. (0.25 mg/g body weight) and allowed to circulate for 5 -10 min to quantifiy albumin leakage as a measure of plasma protein extravasation. E) image of a representative mesenteric vessel containing FITC-conjugated albumin. Average fluorescence intensity in three areas of equal size was measured: inside the vessel (Fl_{in}), outside the vessel (Fl_{out}) and background fluorescence in an area with no obvious leakage (bk). Scale bar = 20 µm. F) Albumin leakage at 0, 20, 60 or 120 min post LPS was determined as: $[(Fl_{out} \text{ x bk})/(Fl^{in} \text{ x bk})]$ x 100 %. Data are expressed as mean ± SEM. n = 5 - 6 mice/group. $^{***}p < 0.001$ vs. saline vehicle-treated counterpart.

Figure 2. Rapid effects of different inflammagens on mesenteric leukocyte-endothelial cell interactions. Mice were treated with LPS (10 µg/mouse, i.p.), zymosan (500 µg/mouse, i.p.), ammonium thioglycollate (250 µl of a 3 % solution, i.p.) or saline vehicle. After 20 min, the mesentery was exteriorised under anaesthesia for visualisation of post-capillary venules by IVM. Leukocyte-endothelial cell interactions were quantified in terms of A) leukocyte rolling velocity (expressed as V_{WBC}), B) number of adherent (stationary for ≥30 s) leukocytes per 100 µm length and C) number of emigrated leukocytes per 100 x 50 μm^2. D) FITC-conjugated albumin was injected i.v. (0.25 mg/g body

weight) and allowed to circulate for 5 -10 min to quantifiy albumin leakage ([(Fl_{out} x bk)/(Fl^{in} x bk)] x 100 %)) as a measure of plasma protein extravasation. Data are mean ± SEM. n = 5 - 6 mice/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. saline vehicle-treated counterpart and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. LPS -treated counterpart.

Figure 3. Neutrophils are the predominant cell type in the inflamed murine mesentery. Mice were treated with LPS (10 µg/mouse, i.p.) or saline vehicle for 0, 20, 60 or 120 min. A) Hematoxylin and eosin (H & E) staining of the mesentery shows adherent neutrophils (aN) and emigrated neutrophils (eN) following LPS stimulation. B) MPO activity was measured in the mesenteric tissue samples. Data are mean ± SEM. n = 5 - 6 mice/group. $^{***}p < 0.001$ vs. saline vehicle-treated counterpart.

Figure 4. Mesenteric mast cell activation and effects on leukocyte-endothelial cell interactions. A) Mice were treated with LPS (10 µg/mouse, i.p.) or saline vehicle. After 20 min, the mesentery was exteriorized and superfused with bicarbonate buffered saline containing 0.001% ruthenium red, which is selectively taken up by activated mast cells. Images of the mesentery were recorded under a X10 objective for 15 min, after which time the mast cell destabilizing agent CMP 48/80 (1 µg/ml) was added to the superfusion buffer. Dye uptake expressed at each timepoint as % of the maximum intensity reached after complete stimulation with CMP 48/80, 25 min post exteriorization or after 10 min superfusion with CMP 48/80. (B,C,D) Mice were treated with vehicle (saline i.p.), sodium cromolyn (CRO, 10 mg/kg i.p.) or $AnxA1_{Ac2\text{-}26}$ (100 µg/mouse i.v.), 15 min prior to injection of LPS (10 µg/mouse i.p.). After 20 min, the mesentery was exteriorized for visualization of post-capillary venules by IVM. Leukocyte-endothelial cell interactions were quantified in terms of B) number of adherent (stationary for ≥30 s) leukocytes per 100 µm length, C) number of emigrated leukocytes per 100 x 50

μm^2 and D) FITC-conjugated albumin was injected i.v. (0.25 mg/g body weight) and allowed to circulate for 5 -10 min to quantifiy albumin leakage ([(Fl_{out} x bk)/(Fl^{in} x bk)] x 100 %)) as a measure of plasma protein extravasation. Data are expressed as mean ± SEM. n = 5 - 6 mice/group. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. corresponding saline vehicle-treated counterpart.

Figure 5: MCs mediate early neutrophil recruitment in inflammation via Fpr2/ALX. Supernatants from purified MC stimulated with saline or LPS (1 μg/ml) for 4 h at 37 °C with 5% CO_2 atmosphere were added to A) a chemotaxis chamber to ascertain the ability of the supernatant act as a neutrophil chemoattractant or directly to B) neutrophils to measure whether the supernatant could induce AnxA1 release from neutrophils. MC were also treated for 20 min with saline, LPS (1 μg/ml), or LPS + CMP 48/80 (10 μg/ml) with or without the addition of cromolyn sodium (CRO) and supernatants were assayed using ELISAs for C) histamine and D) AnxA1 release, or were added to E) isolated murine neutrophils which had been labelled with calcein AM and pre-incubated with saline, the FPR pan antagonist Boc2 (20 μM) or the FPR2 specific antagonist WRW4 (10 μM) for 10 min. The neutrophil-MC supernatant cocktail was incubated together for 10 min prior to adding to a chemotaxis plate for 20 min with fMLP in the lower compartment. Data are expressed as mean ± SEM of 3 experiments with n = 5 mice/group or 3 experiments (chemotaxis). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. saline vehicle-treated counterpart. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. vehicle + LPS + CMP 48/80. $^{\S}p < 0.05$ and $^{\S\S\S\S}p < 0.0001$ vs. neutrophil + LPS + CMP 48/80. $^{\&\&\&}p < 0.001$ and $^{\&\&\&\&}p < 0.0001$ vs. neutrophil + vehicle + LPS + CMP 48/80 + CRO

Figure 6: Schematic overview of the important anti-inflammatory role that MC play in early neutrophil recruitment. Lipopolysacharide (LPS) activates mast cells (MCs) causing them to release

histamine and pro-inflammatory cytokines, including interleukin-6 (IL-6), which in turn leads to a rapid recruitment of neutrophils, within 20 minutes of LPS administration. MCs are ideally positioned in close proximity to the vasculature to initiate thi early phase of neutrophil recruitment. These recruited neutrophils release factors such as myeloperoxidase (MPO) that also leads to the recruitment of more neutrophils. Mast stabilizing drugs, such as cromolyn sodium destabilizes MCs and causes the down regulation of histamine and the release of annexin A1 (AnxA1). MC AnxA1 acts upon the Fpr2/ALX on neutrophils that have arrived within the stimulated tissue, to help promote the anti-inflammatory effect. This demonstrates a positive role for the MC in tissue inflammation via the Fpr2/ALX pathway.

TABLES

Table 1. Hemodynamic parameters in the murine mesenteric microcirculation. Mice were treated with LPS (10 µg/mouse, i.p.) or vehicle saline (50 µl/mouse, i.p.). Vessel diameter and wall shear rate (WSR) of vessels analyzed in Figure 1. Data are mean ± SEM. n = 5 mice/group. $^{***}p < 0.001$ vs. saline at corresponding time-point.

Table 2 Altered levels of pivotal pro-inflammatory cytokines in serum post endotoxin challenge. Mice were treated with LPS (10 µg/mouse, i.p.) or vehicle saline (50 µl/mouse, i.p.). Blood was taken by cardiac puncture 0, 20, 60 and 120 min after the injection and plasma was assayed by enzyme-linked immunosorbent assay (ELISA) for TNF-α, IL-1β and IL-6. Data are mean ± SEM. n = 5 mice/group. $^{***}p < 0.001$ vs. saline at corresponding time-point. A value of 0 indicates levels below the detectable limit of the kit (31.5 pg/ml).

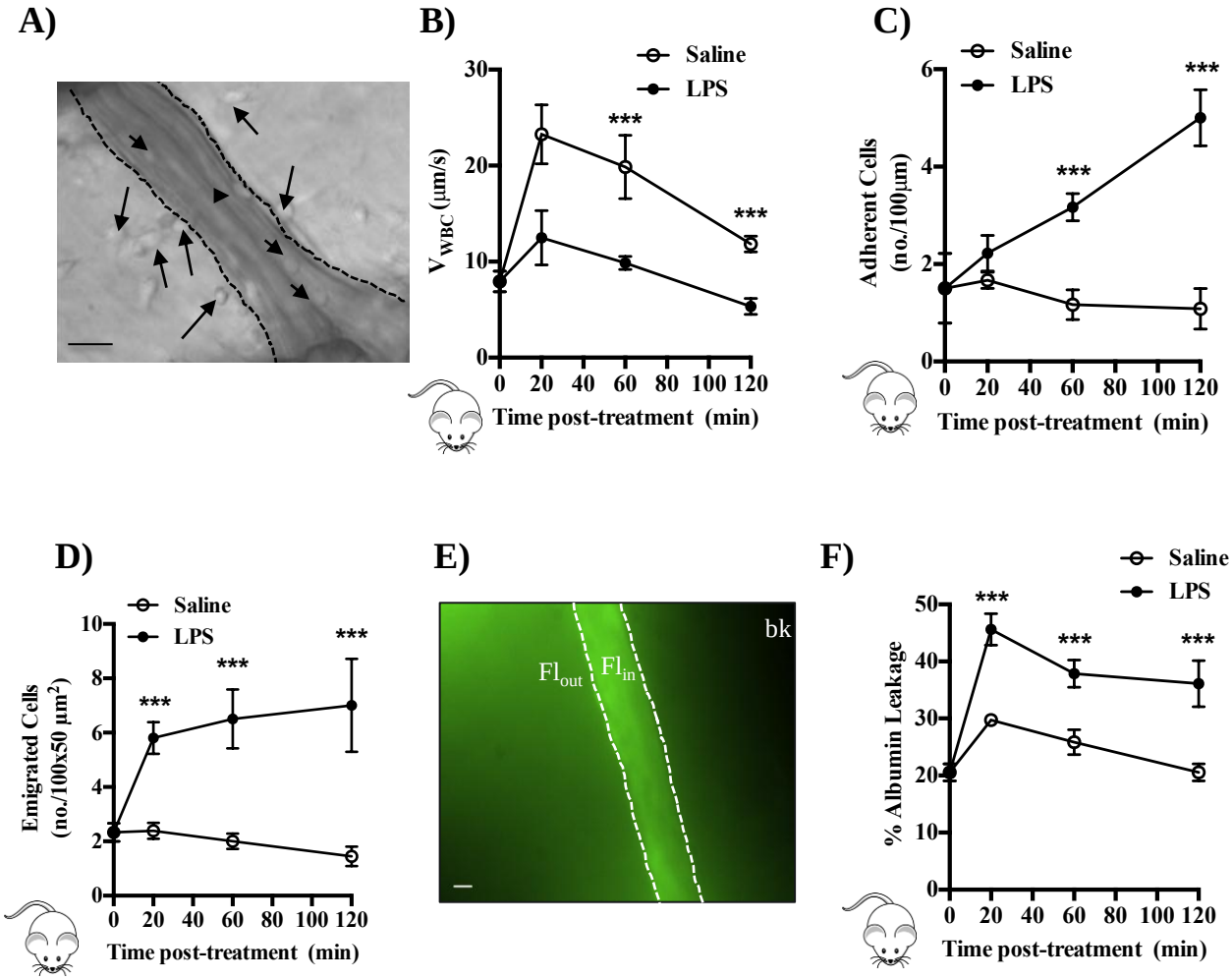

A)
B)
Saline
LPS
V_{WBC} (μm/s)
Time post-treatment (min)
C)
Adherent Cells (no./100μm)
D)
Emigrated Cells (no./100x50 μm²)
E)
bk
Fl_{out}
Fl_{in}
F)
% Albumin Leakage
***

Figure. 1

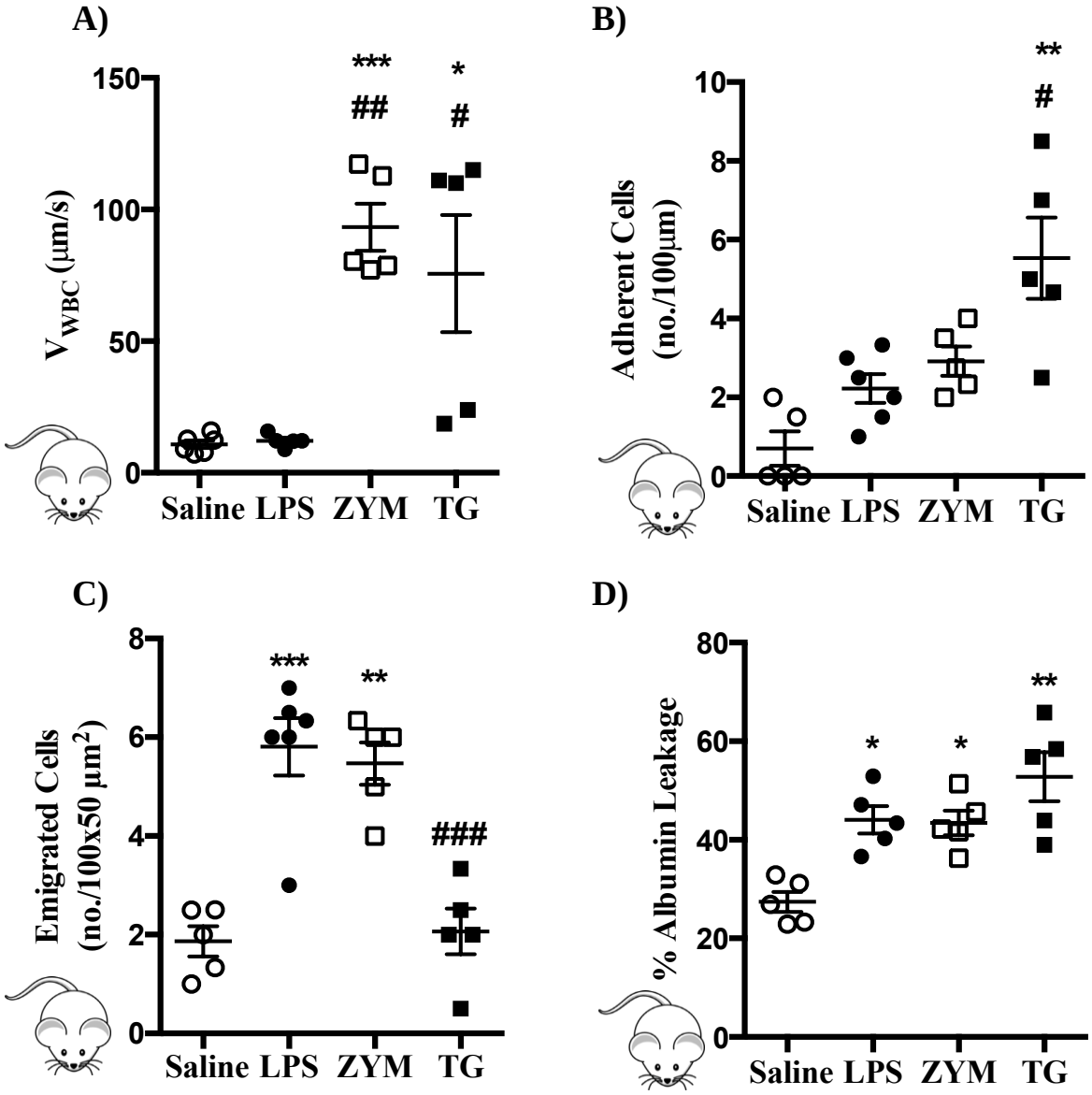

A)
V_{WBC} (μm/s)
150
100
50
0
***
##
*
#
Saline LPS ZYM TG
B)
Adherent Cells (no./100μm)
10
8
6
4
2
0
**
#
Saline LPS ZYM TG
C)
Emigrated Cells (no./100x50 μm²)
8
6
4
2
0
***
**
###
Saline LPS ZYM TG
D)
% Albumin Leakage
80
60
40
20
0
*
*
**
Saline LPS ZYM TG

Figure. 2

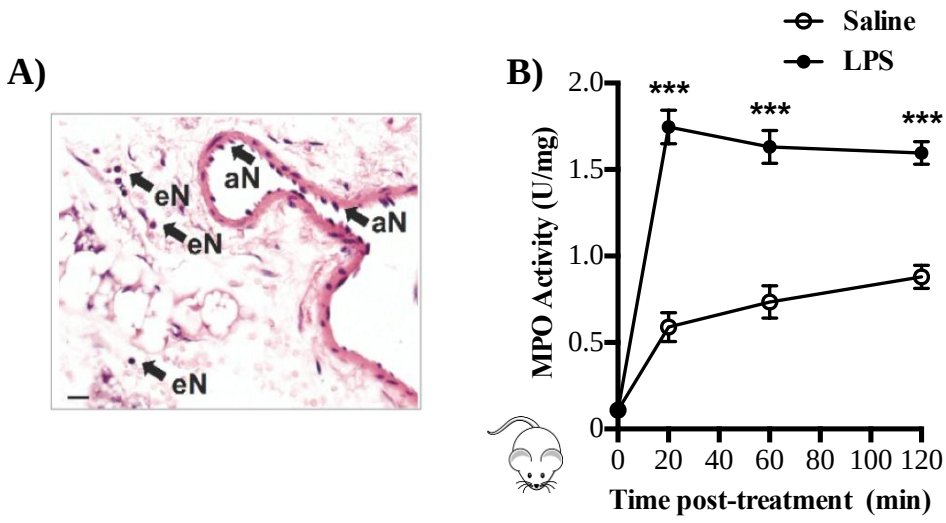

A)
aN
eN
eN
aN
eN
B)
Saline
LPS
MPO Activity (U/mg)
2.0
1.5
1.0
0.5
0
***
***
***
0 20 40 60 80 100 120
Time post-treatment (min)

Figure. 3

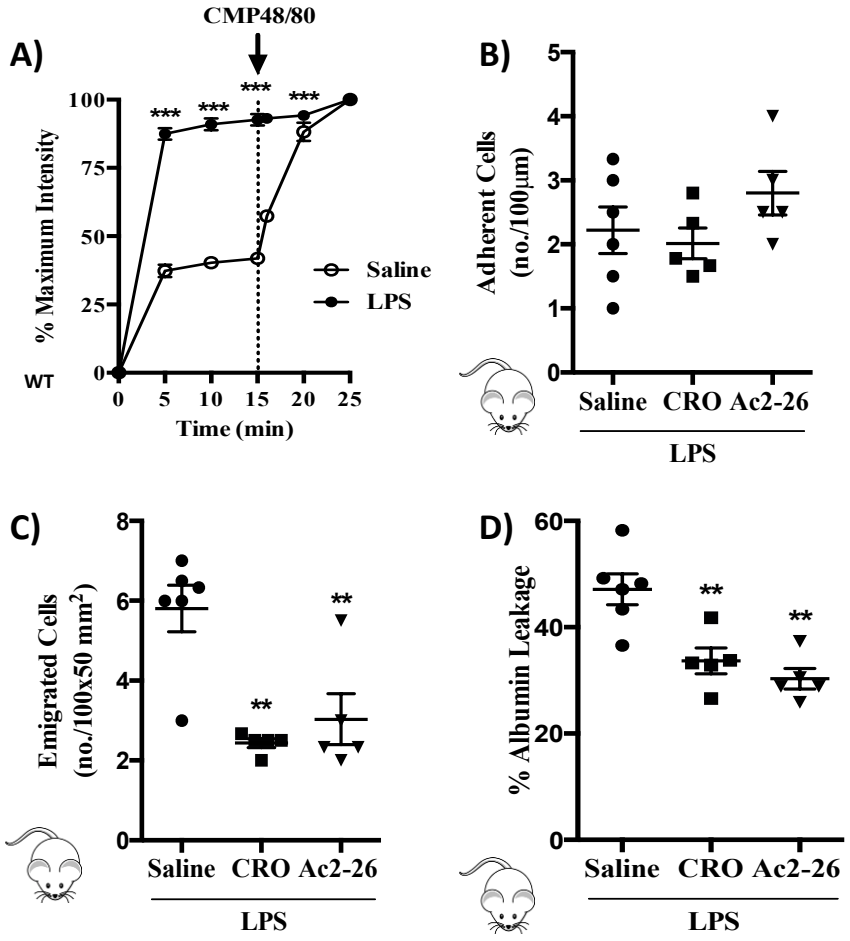

A)
CMP48/80
% Maximum Intensity
Time (min)
Saline
LPS
WT
***
B)
Adherent Cells (no./100µm)
Saline
CRO
Ac2-26
LPS
C)
Emigrated Cells (no./100x50 mm²)
**
D)
% Albumin Leakage
**

Figure. 4

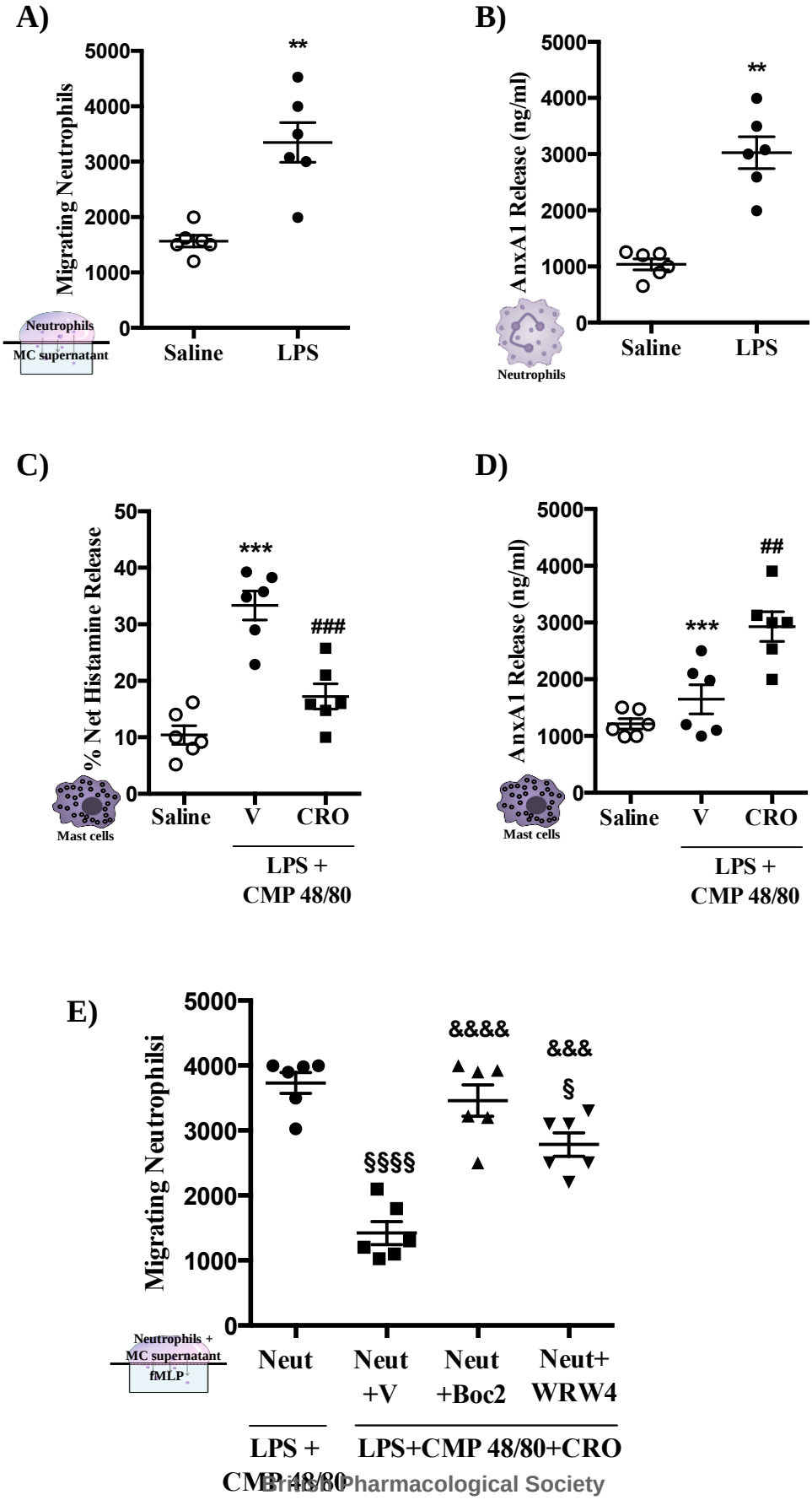
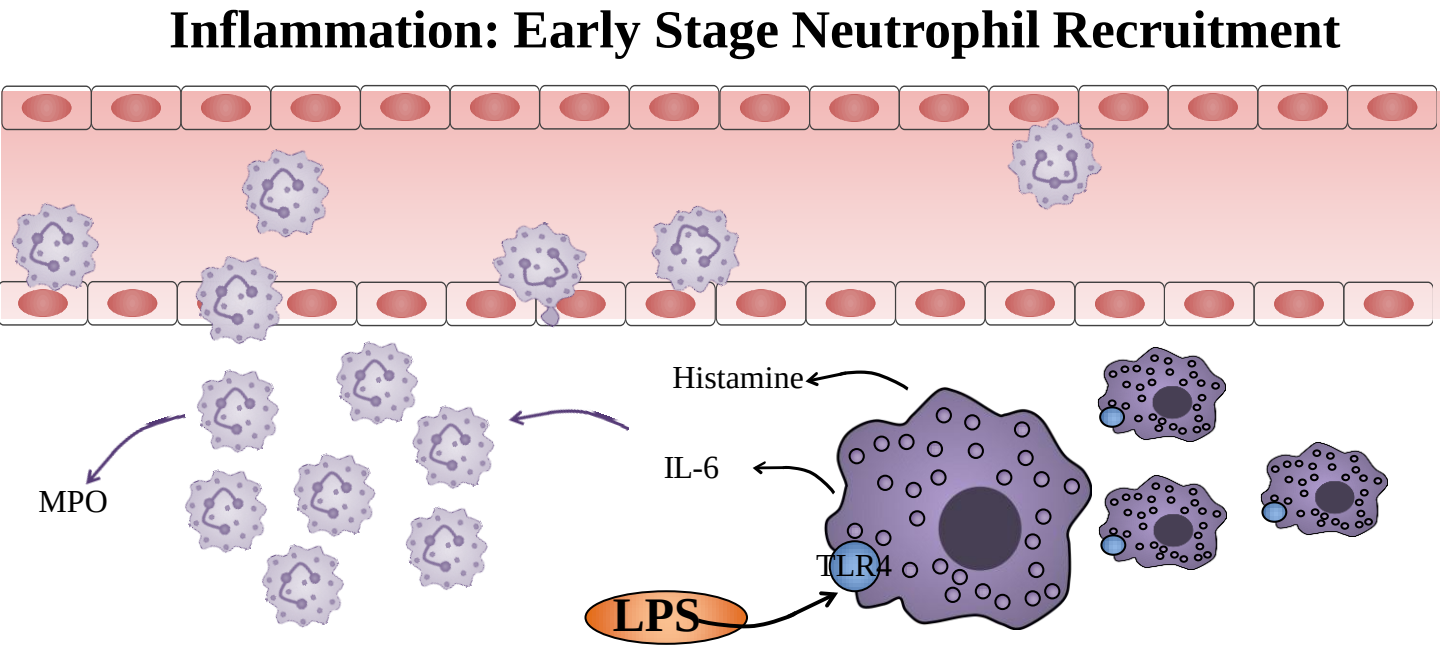


Figure. 5


Inflammation: Early Stage Neutrophil Recruitment
Histamine
IL-6
MPO
TLR4
LPS

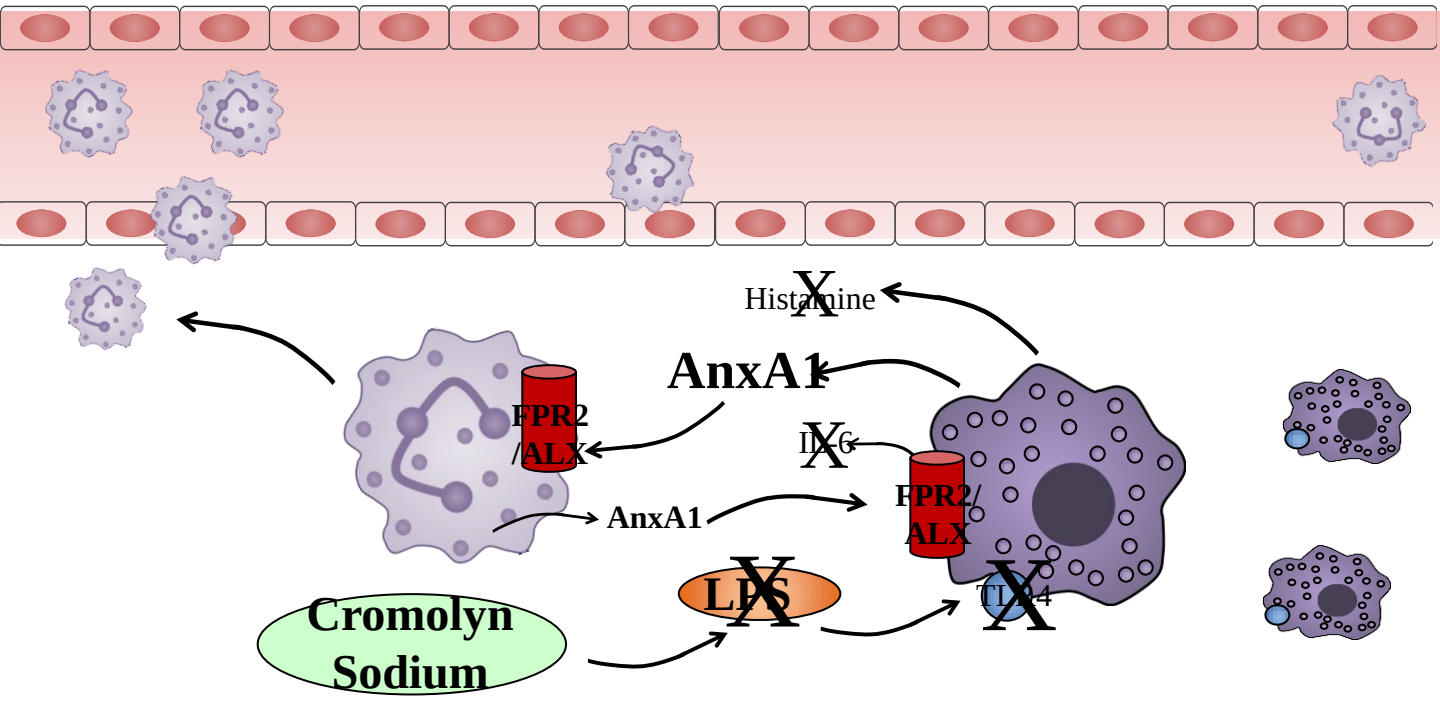

MCs mediate early neutrophil recruitment via FPR2/ALX
Histamine
AnxA1
FPR2
/ALX
IL-6
AnxA1
FPR2/
ALX
LPS
TLR4
Cromolyn
Sodium

Figure. 6

Table 1. Hemodynamic parameters in the mesenteric microcirculation of WT mice.

Genotype	Treatment	Time post LPS (min)	Vessel diameter (μm)	Wall shear rate (per s)
	Saline	0	28.4 ± 2.2	546 ± 12.6
		20	25.2 ± 3.1	529 ± 11.4
		60	28.8 ± 2.1	556.8 ± 9.5
		120	30.2 ± 1.9	558 ± 9.5
	LPS	0	26.8 ± 2.9	531 ± 16.3
		20	30.1 ± 2.1	532 ± 13.6
		60	26.8 ± 2.1	450 ± 31.4***
		120	28.2 ± 1.4	334 ± 34.0***

LPS dose administered = 10 μg/mouse. Data are mean ± SEM. n = 5 mice/group.

***$p < 0.001$ vs. saline at corresponding time-point.

Table 2. Altered levels of pivotal pro-inflammatory cytokines in serum post endotoxin challenge.

Time post-treatment (min)	TNF-α (pg/ml)		IL-1β (pg/ml)		IL-6 (pg/ml)	
	Saline	*LPS*	*Saline*	*LPS*	*Saline*	*LPS*
0	0	0	0	0	0	0
20	0	0	0	0	0	0
60	43.71 ± 4.4	304.6 ± 29.2***	91.9 ± 9.3	321.7 ± 29.6***	60.7 ± 11.3	157.1 ± 14.8***
120	88.7 ± 4.8	663.3 ± 35.4***	84.3 ± 4.8	702.7 ± 33.9***	55.9 ± 6.6	513.8 ± 49.7***

Data are mean ± SEM. $n = 5$ mice/group. $^{***}p < 0.001$ vs. saline at corresponding time-point. A value of 0 indicates levels below the detectable limit of the kit (31.5 pg/ml).