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Key points:

- Murine neutrophils can be stimulated by LPS to express de-novo ICAM-1 *in vitro* and in murine models of endotoxemia *in vivo*.
- ICAM-1 expressing neutrophils exhibited increased phagocytosis and ROS generation *in vitro* and ICAM-1 deficient neutrophils were defective in phagocytosis in murine models of endotoxemia.

Abstract

Intracellular adhesion molecule-1 (ICAM-1) is a transmembrane glycoprotein expressed on the cell surface of numerous cell types such as endothelial and epithelial cells, vascular smooth muscle cells and certain leukocyte sub-sets. With respect to the latter, ICAM-1 has been detected on neutrophils in several clinical and experimental settings but little is known about the regulation of expression or function of neutrophil ICAM-1. In this study we report on the de-novo induction of ICAM-1 on the cell surface of murine neutrophils by lipopolysaccharide (LPS), TNF and zymosan particles *in vitro*. The induction of neutrophil ICAM-1 was associated with enhanced phagocytosis of zymosan particles and ROS generation. Conversely, neutrophils from ICAM-1 deficient mice were defective in these effector functions. Mechanistically, ICAM-1-mediated intracellular signalling appeared to support neutrophil ROS generation and phagocytosis. *In vivo,* LPS-induced inflammation in the mouse cremaster muscle and peritoneal cavity led to ICAM-1 expression on intravascular and locally transmigrated neutrophils. The use of chimeric mice deficient in ICAM-1 on myeloid cells demonstrated that neutrophil ICAM-1 was not required for local neutrophil transmigration but supported optimal intravascular and extravascular phagocytosis of zymosan particles. Collectively the present results shed light on regulation of expression and function of ICAM-1 on neutrophils and identify it as an additional regulator of neutrophil effector responses in host defence.

Introduction

ICAM-1 (CD54) is a member of the immunoglobulin (Ig)-like gene superfamily composed of an extracellular domain containing 5 Ig-like structures, a transmembrane domain and a short cytoplasmic tail of 28 amino acids.[1-3] It is a key adhesion molecule with significant signalling properties that has been associated with numerous cellular responses such as cell adhesion, migration and aggregation.[4-6] ICAM-1 is constitutively expressed on the surface of a wide range of cells including endothelial and epithelial cells, smooth muscle cells, pericytes, fibroblasts and keratinocytes.[4;5;7-9] The expression of ICAM-1 is primarily regulated in a transcriptional manner, notably by inflammatory stimuli such as the cytokines IL-1β and TNF and by LPS.[4;9;10] For example, these stimuli can enhance the constitutive expression of endothelial cell ICAM-1 and promote leukocyte-endothelial cell adhesion and trafficking via interactions of ICAM-1 with its key leukocyte ligands, the integrins LFA-1 and Mac-1.[8;11;12] Ligation of endothelial cell ICAM-1 can trigger elevations in cytoplasmic Ca^{2+}, activation of myosin contractility, PKC and of the small GTPases (e.g. Rho, Rac and Rap1).[6;10] A number of cytosolic and adaptor proteins, such as α-actinins, ezrin, cortactin and filamin B, also interact with the C-terminal domain of endothelial cell ICAM-1 and contribute to localised cytoskeletal re-arrangements and leukocyte-endothelial interactions.[13] How such events are linked in endothelial cells, and details of ICAM-1 signalling in different cell types are not fully understood, but it is generally accepted that the cytoplasmic tail of the molecule plays a key role in supporting ICAM-1-mediated responses.

In addition to being expressed on tissue-resident cells, ICAM-1 is expressed on most leukocyte sub-sets such as activated lymphocytes and monocytes. The best documented role of leukocyte ICAM-1 is in the formation of immune synapses between T-cells and antigen presenting or NK cells and their targets.[14] These responses are primarily mediated by ICAM-1-LFA-1 interactions.[14] With respect to neutrophils, ICAM-1 is generally absent or expressed at very low levels on circulating blood cells in humans and mice[15-18] though elevated levels have been reported in a limited number of clinical and experimental scenarios. For example, ICAM-1 has been detected on blood and peritoneal neutrophils in patients with bacterial peritonitis,[19] on blood and nasopharyngeal aspirated neutrophils in infants with respiratory syncytial virus,[20] on blood neutrophils post low dose intravenous (i.v.) endotoxin[21] and on blood and bronchoalveolar lavage neutrophils from septic and sarcoidosis patients.[22] In animal models, immunisation of mice with *P. aeruginosa* can also upregulate neutrophil ICAM-1 *in vivo*[18] and neutrophil reverse transendothelial cell migration has been associated with increased neutrophil ICAM-1 expression *in vitro* and *in vivo* with respect to human and mouse neutrophils.[17;23] Furthermore, there are indications that G-CSF, GM-CSF, LPS, TNF, bacterial lipoprotein and *S. aureus* can induce expression of ICAM-1 on

human neutrophils *in vitro*.[16;24-27] In terms of its physiological role, neutrophil ICAM-1 has been linked to cellular aggregation, increased generation of reactive oxygen species (ROS)[26;28] and in supporting interactions with other components of the immune system.[21] Despite the above scant reports, little is known about the mechanisms through which ICAM-1 is expressed on neutrophils and its potential pathophysiological functions. In the present study we demonstrate the ability of murine neutrophils to upregulate ICAM-1 in a stimulus-specific manner both *in vitro* and *in vivo* with LPS being identified as an effective inducer of neutrophil ICAM-1 mRNA. Functionally, neutrophil ICAM-1 was found to be important for enhanced neutrophil effector functions, with ICAM-1 deficient neutrophils exhibiting significantly reduced levels of phagocytosis *in vitro* and in murine models of endotoxemia. The mechanism through which neutrophil ICAM-1 mediated phagocytosis appeared to be linked to ICAM-1-fibrinogen interactions and ICAM-1-mediated intracellular signalling involving the tyrosine kinase Syk. Collectively, the present findings shed significant light on the regulation and function of neutrophil ICAM-1 and suggest that in addition to its expression on endothelial cells and lymphocytes, neutrophil ICAM-1 contributes to the host's defence mechanism against pathogens.

Materials and Methods

For detailed methodology see Supplementary Materials.

Animals: Male wild type (WT), ICAM-1 deficient (KO),[29] or heterozygous LysM-eGFP mice on a C57BL/6 background were used. LysM-eGFP[30] mice are referred to as WT-LysM-eGFP, to distinguish them from WT mice. All experiments were performed under the UK Home Office legislation for the protection of animals.

Generation of chimeric mice: A mixture of WT-LysM-eGFP and ICAM-1 KO bone marrow was injected intravenously (i.v.) to irradiated WT mice to create chimeric mice that expressed ICAM-1 on the vasculature with ICAM-1 KO or WT-LysM-GFP leukocytes.[31]

In vivo inflammatory models: Animals were injected with intrascrotal (i.s.) or intraperitoneal (i.p.) LPS or IL-1β and after 4 hrs blood, peritoneal lavage and tissue samples were collected. In some cases, fluorescent zymosan-Texas-Red (ZymTR) was injected (i.p. or i.v.) 15 minutes prior to tissue collection to quantify *in vivo* phagocytosis. Cremaster muscles and lungs were labelled with fluorescent mAbs against PECAM-1 (endothelial cell junctions), S100a9 (neutrophils), lectin (vasculature) and ICAM-1, and analysed by confocal microscopy. Leukocytes from blood, enzymatically digested lung tissue or peritoneal lavage were analysed by flow cytometry (see below).

Quantification of ROS and phagocytosis: Whole blood from WT or ICAM-1 KO mice was stimulated with a panel of inflammatory mediators for 4 hrs. The ROS probe dihydrorhodamine-123 (DHR), ZymTR and all pharmacological interventions were added to blood samples as detailed.

Flow Cytometry: Blood, peritoneal lavage or lung tissue digest leukocytes were labelled with the fluorescent dead cell nuclear marker DAPI and fluorescent mAbs against CD45, Ly6G, ICAM-1, or isotype controls, or the ROS probe DHR or ZymTR prior to analysis by flow cytometry.

Confocal microscopy: Fluorescently labelled cremaster muscles, lung tissues or isolated leukocytes were viewed using a Leica-SP5 confocal microscope incorporating a 20× water-dipping objective (NA 1.0). Quantifications were carried out using Imaris (Bitplane) or Leica LASF-Lite software.

Quantitative RT-PCR: Neutrophils were purified by FACS. The fold change in ICAM-1 mRNA was quantified using GAPDH as a housekeeping gene.

Western Blot: Naïve or LPS stimulated neutrophils were purified and treated with protease inhibitor before lysis. ICAM-1 and β-actin expression was detected by Western Blot.

Statistical analysis: Results are presented as mean ± s.e.m. Statistical significance was assessed by the Student t-test or by one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison test. P values below 0.05 were considered significant.

Results

ICAM-1 is upregulated on mouse neutrophils in a stimulus-specific manner *in vitro*

Whilst a number of studies have reported on the presence of ICAM-1 on neutrophils,[19;20;22] little is known about the regulation of this expression. Analysis of mouse blood leukocytes by flow cytometry indicated a low percentage of ICAM-1 positive neutrophils. This was not changed following *in vitro* stimulation of whole blood with a number of neutrophil chemotactic stimuli (LTB_4, KC and fMLP) or with the cytokine IL-1β (4 hrs stimulation at 37℃ for all mediators; Fig 1A). In contrast, LPS, TNF and zymosan particles significantly increased the percentage of ICAM-1 expressing neutrophils (Fig 1A). The lack of ICAM-1 staining in samples from ICAM-1 KO mice confirmed the labelling specificity. Of note, WT and ICAM-1 KO neutrophils showed similar levels of enhanced CD18 expression in response to LPS (data not shown).

The effect of LPS was investigated further and shown to induce ICAM-1 on neutrophils in terms of relative fluorescence intensity (RFI) and also in a dose-dependent manner (Figs 1B and 1C). LPS-induced expression of ICAM-1 on neutrophils was time-dependent and a significant effect was noted at 3 hrs post stimulation (Fig 1D). The observed slow expression suggested that this ICAM-1 was not derived from preformed intracellular stores and indeed no evidence for intracellular expression of ICAM-1 in permeablised unstimulated neutrophils was observed (Fig 1E). Quantitative RT-PCR analysis of unstimulated and LPS-stimulated purified neutrophils indicated low levels of ICAM-1 mRNA in unstimulated cells that was significantly elevated by LPS, providing evidence for *de novo* generation of ICAM-1 (Fig 1F). Western Blot analysis of unstimulated and LPS-stimulated neutrophils, treated with a protease inhibitor, confirmed that naïve cells have low or absent levels of ICAM-1 (Fig 1G).

Collectively these results demonstrate that murine neutrophils can be stimulated to express cell surface ICAM-1 in a stimulus-specific manner via induction of ICAM-1 mRNA and *de novo* generation of ICAM-1 protein.

Neutrophil ICAM-1 expression facilitates enhanced neutrophil effector functions

The potential functional implications of LPS-induced neutrophil ICAM-1 were next investigated. As the ICAM-1 ligand Mac-1 is a key regulator of neutrophil phagocytosis, we hypothesised that neutrophil ICAM-1 might also facilitate this process. To address this possibility neutrophil phagocytosis was quantified using fluorescent zymosan particles (ZymTR). LPS stimulation of WT blood samples significantly increased the percentage of ZymTR positive neutrophils ($ZymTR^{pos}$), as compared to saline-treated samples (Fig 2A and B). This response was significantly suppressed in samples from ICAM-1 KOs (Fig 2B). Use of an ImageStream platform, combining fluorescence microscopy with high-throughput flow cytometry, indicated that in both genotypes the majority of ZymTR-associated neutrophils had internalised the ZymTR particles within 15 minutes (ie ~97%), confirming that this assay quantifies phagocytosis of ZymTR, rather than surface decoration. Neutrophil ICAM-1 also appeared to support ROS generation. Specifically, LPS-stimulated WT samples showed significantly higher DHR signal, as compared to saline-treated samples, and this was significantly reduced in samples from ICAM-1 KO mice (Fig 2C). Furthermore, ICAM-1 positive neutrophils exhibited a greater DHR signal than ICAM-1 negative cells (Fig 2D). Interestingly, across the panel of neutrophil stimuli used in Fig 1A a correlation between ICAM-1 upregulation and ROS generation was noted with LPS and TNF inducing ICAM-1 expression and ROS generation, while IL-1β, fMLP and KC did not (Fig 2E). Collectively, these results demonstrate the involvement of neutrophil ICAM-1 in generation of intracellular

ROS and report on a previously unknown function of neutrophil ICAM-1 as a facilitator of phagocytosis.

Mechanism of neutrophil ICAM-1-mediated enhanced effector function?

Having identified a role for neutrophil ICAM-1 in phagocytosis and ROS generation, the potential associated mechanisms were addressed. To test the hypothesis that cell surface ICAM-1 may be involved in uptake of zymosan particles the effect of an anti-ICAM-1 mAb that inhibits ICAM-1 integrin binding[32;33] on LPS-induced phagocytosis was examined. In contrast to the effects seen under conditions of ICAM-1 genetic deletion (Fig 2B), surface blockade of ICAM-1 had no impact on zymosan phagocytosis (Fig 3A). Using the same protocol, as expected an anti-Mac-1 mAb significantly inhibited LPS-induced zymosan phagocytosis with a similar level of suppression in both WT and ICAM-1 KO samples. As no role for ICAM-1-integrin binding was seen, we hypothesised that neutrophil ICAM-1 may interact with the other important ICAM-1 ligand fibrinogen and hence support uptake of fibrinogen-coated zymosan particles. To test this possibility, we employed a peptide mimicking the gamma domain of fibrinogen that competitively inhibits ICAM-1-fibrinogen interactions[34;35]. Addition of this peptide to LPS-stimulated WT samples led to a small but significant inhibition of ZymTR phagocytosis (Fig 3B). Importantly, the peptide blocker had no effect on phagocytosis by LPS-stimulated ICAM-1 KO neutrophils (Fig 3B), indicating the specificity of the inhibitor for ICAM-1-mediated responses.

Since cell surface binding capability of neutrophil ICAM-1 revealed a modest role in regulation of phagocytosis, we hypothesised that the principal role of neutrophil ICAM-1 in regulating neutrophil effector functions may reside in its signalling capability. To test this, the effect of a cell permeant peptide that mimics the intracellular C-terminal domain of ICAM-1, widely used as a blocker of ICAM-1-mediated intracellular signalling,[36;37] was investigated. Pre-treatment of WT blood samples with the peptide led to significant inhibition of LPS-induced phagocytosis of zymosan particles (Fig 3C). Importantly, the peptide had no effect on phagocytosis responses detected in ICAM-1 KO samples (Fig 3C), indicating specificity of the peptide for ICAM-1-mediated events.

As the cytoplasmic tyrosine kinase Syk has been linked to both ICAM-1 intracellular signalling[38] and phagocytosis,[39;40] the potential role of this kinase in LPS-induced phagocytosis was investigated using the inhibitor Piceatannol. Pre-treatment of blood samples with Piceatannol dose-dependently inhibited LPS-induced ZymTR uptake in WT neutrophils. ICAM-1 KO neutrophils were less sensitive to Piceatannol, indicating that Syk mediated mechanisms have an ICAM-1 dependent component (Fig 3D). Furthermore,

antibody cross-linking of ICAM-1 that is known to activate intracellular signalling,[10] enhanced LPS-stimulated ROS generation (Fig 3E), providing additional indication for the involvement of ICAM-1-signalling in regulation of neutrophil effector functions.

Collectively these data provide evidence to suggest that ICAM-1 expression enhances neutrophil effector functions via interactions with fibrinogen and through intracellular signalling involving activation of Syk mediated pathways.

ICAM-1 is expressed on neutrophils in models of endotoxemia *in vivo*

Having found that LPS induces surface expression of ICAM-1 on neutrophils and that this expression supports enhanced neutrophil effector functions *in vitro*, we next sought to investigate the expression and potential functions of neutrophil ICAM-1 *in vivo*. For this purpose, two murine models of local endotoxemia, namely LPS-stimulated cremaster muscles (i.s.) and LPS-induced peritonitis (i.p.), were employed. For comparison, the same models, as driven by locally injected IL-1β, a stimulus that did not induce neutrophil ICAM-1 expression *in vitro* (Fig 1A), were also investigated. Locally injected LPS and IL-1β induced significant neutrophil infiltration in the cremaster muscle and peritoneal cavity (Fig 4A-C). Furthermore, in both models, LPS-induced tissue infiltrated neutrophils exhibited significant expression of ICAM-1 (Fig 4D-F). Of note, neutrophils accumulating in response to locally injected IL-1β showed very little or no ICAM-1 expression (Figs 4C-D). In both models, local LPS (but not IL-1β) appeared to exert a systemic effect in that there was a significant increase in the proportion of neutrophils in blood and in enzymatically digested lung tissues (Fig 4G). Importantly, i.v. administration of a fluorescently-labelled anti-Ly6G antibody (used to label all intravascular neutrophils) prior to tissue collection, indicated that in both naïve and LPS treated animals >90% of neutrophils in the lung digest were intravascular. As found with the tissue infiltrated cells, in the LPS-driven reactions (but not IL-1β), an increase in the percentage of blood ICAM-1 positive neutrophils was detected (Fig 4H). Lung intravascular neutrophils also exhibited increased ICAM-1 expression following intraperitoneal LPS (data not shown).

Collectively these results demonstrate that vascular and tissue infiltrated neutrophils can express ICAM-1 on their cell surface in a stimulus-dependent manner.

Neutrophil ICAM-1 does not support neutrophil transmigration *in vivo*

To investigate the functional role of neutrophil ICAM-1 *in vivo* we generated chimeric mice expressing ICAM-1 on their vasculature but deficient in neutrophil ICAM-1. Lethally irradiated WT mice were reconstituted with a mixture of WT bone-marrow hematopoietic cells from

LysM-eGFP Ki mice (express GFP-labelled myeloid cells) and GFP negative ICAM-1 KO mice. There was an average peripheral blood ratio of 60:40 WT:KO neutrophils in these animals (Supplementary figure 1A). This model provided a valuable tool for directly investigating the role of neutrophil ICAM-1 in neutrophil transmigration *in vivo*. In the cremaster muscle and peritonitis models, LPS-induced local neutrophil infiltration was quantified by confocal microscopy and flow cytometry, respectively, enabling WT and KO neutrophils to be easily distinguished in the same samples (i.e. WT neutrophils were double positive for GFP and S100a9 or Ly6G whilst ICAM-1 KO neutrophils were only S100a9 or Ly6G positive). With this rigorous approach, the same WT:KO ratio was seen in the peritoneal cavity as in the peripheral blood (Supplementary figure 1A). When the numbers of extravasated cells were normalised to the blood ratio of individual animals no significant difference in tissue infiltration of WT or ICAM-1 KO neutrophils was noted (Fig 5A-C). These results demonstrate that neutrophil ICAM-1 is not required for acute neutrophil infiltration in response to local LPS.

ICAM-1 facilitates neutrophil phagocytosis *in vivo*

As our *in vitro* studies identified phagocytosis as a principal effector function associated with neutrophil ICAM-1, we sought to investigate this association *in vivo*. For this purpose, we took advantage of the chimeric mice detailed above. The mice were subjected to LPS-induced peritonitis, a model that induces blood neutrophilia (Fig 4G), tissue infiltration of neutrophils (Fig 4C) and ICAM-1 expression on intravascular and peritoneal neutrophils (Fig 4G and 4F). ZymTR was given i.v. or i.p. 15 minutes prior to sample and/or tissue collection and percentage of $ZymTR^{pos}$ WT-LysM-eGFP and ICAM-1 KO neutrophils was quantified by flow cytometry. This technique enabled us to directly compare WT and ICAM-1 KO neutrophils in the same samples, over-riding potential animal variations. With this approach, WT peritoneal neutrophils showed a robust phagocytosis response that was significantly reduced in ICAM-1 KO cells (Fig 6A).

In contrast, blood neutrophils exhibited a low frequency of ZymTR uptake regardless of genotype or stimulation (Fig 6B). Interestingly, WT lung digest neutrophils, that represent predominantly pulmonary vascular neutrophils, showed a marked phagocytosis response in control animals that was further increased in LPS-stimulated mice (Fig 6B). This suggests that the slow blood flow of the pulmonary vasculature supports greater contact between circulating neutrophils and zymosan particles. Lung digest ICAM-1 KO neutrophils showed a similar level of $ZymTR^{pos}$ neutrophils in saline treated mice, but no increase in phagocytosis was seen in LPS-stimulated ICAM-1 KO animals (Fig 6B). Analysis of these events in terms of the total number of $ZymTR^{pos}$ pulmonary vascular neutrophils showed a similar pattern. In

LPS-stimulated mice the total number of ZymTR^{pos} WT neutrophils increased by 47.9% and in the same animals no such increase was seen with ICAM-1 KO cells (Fig 6C). Interestingly there was a small but significant increase in the proportion of WT neutrophils in the lung tissue digest as compared to blood, suggesting a potential preferential retention of WT cells in the lung vasculature. Confocal microscopy images of lung tissue of LPS-stimulated (i.p) mice illustrate the dense network of small capillaries in lungs and also the notable presence of luminal ZymTR^{pos} neutrophils (Fig 6D). In contrast, the microvessels of the cremaster are much less dense, and show little evidence of luminal ZymTR particles in LPS (i.s.) stimulated tissues (Fig 6D).

Collectively these results indicate that neutrophil ICAM-1 can support enhanced phagocytosis both within the vascular lumen (most notably in the pulmonary vasculature) and in the extravascular tissue.

Discussion

Despite the tremendous interest in the expression and function of endothelial cell ICAM-1 and the co-stimulatory role of ICAM-1 in lymphocytes, little is known about neutrophil expressed ICAM-1. In the present study we identify a stimulus-specific transcriptional regulation of ICAM-1 on murine neutrophils and report on the ability of certain stimuli to induce neutrophil ICAM-1 both *in vitro* and *in vivo*. Functionally, this expression was not essential for neutrophil migration into sites of inflammation but supported enhanced neutrophil ROS generation and phagocytosis. The findings shed light on the mechanisms of expression of neutrophil ICAM-1 and identify it as a novel component of the host's innate immune response to pathogens.

ICAM-1 is not commonly considered as a neutrophil cell surface antigen but it has been detected on neutrophils in numerous infectious and inflammatory clinical settings.[19;20;22] Due to the disparity of reports, a key objective of the present work was to gain a better understanding of the expression and regulation of expression of neutrophil ICAM-1, as studied using murine models. Initial *in vitro* experiments showed that in unstimulated murine blood only ~10% of neutrophils were ICAM-1 positive and that these cells expressed very low levels of the molecule (RFI <2.0). Furthermore, stimulation with a wide-range of neutrophil activating and/or pro-inflammatory mediators (KC, fMLP, LTB_4 and IL-1β) had no impact on neutrophil ICAM-1 expression. However LPS, TNF and zymosan particles induced a significant upregulation of ICAM-1 (~60-80% ICAM-1 positive cells), indicating that this response is stimulus-specific. Of importance, LPS-induced upregulation of ICAM-1 was slow

(~3 hrs) and associated with induction of ICAM-1 mRNA, suggesting that it is transcriptionally regulated. This finding is in line with transcriptional regulation of ICAM-1 expression in other cell types such as endothelial and epithelial cells, pericytes and keratinocytes.[4;7;9] Furthermore, it is now well accepted that neutrophils are not transcriptionally static cells and in fact have the capability of significant *de novo* protein synthesis.[41;42] Finally, our observed stimulus-specific pattern of murine neutrophil ICAM-1 expression is in agreement with reports that have linked human neutrophil ICAM-1 expression with the stimulants LPS and TNF,[21;24;26] bacterial lipoprotein[25] and *S. aureus*[24] *in vitro.*

As the profile of stimuli that induce neutrophil ICAM-1 are pathogen-derived and/or are commonly released under conditions of infections, we hypothesised that elevated neutrophil ICAM-1 may represent a physiological response associated with pathogen clearance. Indeed, whilst neutrophil ICAM-1 has to date been largely investigated in the context of neutrophil-neutrophil adhesion, aggregation[26;28] and neutrophil interactions with other components of the immune response,[21] here we made the novel observation that neutrophil ICAM-1 supports an efficient phagocytosis response and is also associated with enhanced ROS generation. Whilst endothelial and epithelial cell ICAM-1 have been shown to support neutrophil phagocytosis by providing co-stimulatory signals through neutrophil integrins,[43-45] to our knowledge this is the first report on the involvement of neutrophil ICAM-1 in neutrophil phagocytosis. Of note, ICAM-1 KO neutrophils did exhibit a significant increase in phagocytosis after LPS stimulation, indicating that as expected ICAM-1-independent phagocytic pathways such as those involving Mac-1/CR3 or FCγRs are also activated by LPS.[46;47]

Phagocytosis is a complex cellular process that is critical for innate immunity.[46-49] It is an adhesion-dependent response with the phagocytic vacuole or phagosome being formed following association of ligands on the surface of the particle (endogenous constituents of the target particle or serum-derived opsonins) with receptors on the cell surface of the phagocyte.[46;48] Post-binding of the target, actin-driven intracellular events mediate particle engulfment.[46;48] In addressing the mechanism through which neutrophil ICAM-1 mediates neutrophil phagocytosis, the functional role of the extracellular and intracellular domains of ICAM-1 was investigated. An antibody that blocks ICAM-1-integrin interactions had no effect on neutrophil phagocytosis of zymosan particles. However, as the blocking anti-ICAM-1 mAb employed has little or no inhibitory effect on ICAM-1 interactions with its other key ligand, fibrinogen,[50-52] the potential involvement of neutrophil ICAM-1 binding to fibrinogen-coated zymosan particles was a possibility.[2;34] The use of a competitive peptide antagonist of ICAM-1-fibrinogen interaction[35;53] indicated a modest but significant and specific role for fibrinogen

in ICAM-1-mediated neutrophil phagocytosis. Additionally, a membrane penetrating peptide that mimics the intracellular domain of ICAM-1, and has been used to inhibit interactions of ICAM-1 with cytoplasmic accessory proteins,[36;37] inhibited ICAM-1-dependent LPS-stimulated phagocytosis. Collectively, based on our results and published reports we propose that cell surface neutrophil ICAM-1 can mediate phagocytosis possibly independently of leukocyte integrins and possibly via interactions of neutrophil ICAM-1 with fibrinogen-coated particles. This interaction can then trigger ICAM-1-mediated signalling that supports efficient phagocytosis. The precise details of the latter are unclear and dissecting the different components was beyond the scope of the present study. However as the cytoplasmic tyrosine kinase Syk has been linked to phagocytosis by neutrophils and macrophages,[39;40] and is activated by ICAM-1-mediated intracellular signalling in epithelial cells,[38] the role of Syk in ICAM-1-mediated neutrophil phagocytosis was explored. Briefly, the Syk inhibitor Piceatannol induced a dose-dependent suppression of LPS-induced neutrophil phagocytosis, with the maximal effect being similar to that seen under conditions of ICAM-1 deletion. These results suggest a potential role for Syk in cell autonomous neutrophil ICAM-1-mediated phagocytosis. Of note, the majority of studies looking at ICAM-1 intracellular signalling have focussed on endothelial ICAM-1 in the context of leukocyte transmigration.[36;54;55] In such studies, clustering and dimerization of endothelial ICAM-1 via ligation of leukocyte integrins, or cross-linking with antibody coated beads, promote association of the cytoplasmic domain with adaptor proteins and activation of subsequent effector pathways. These include modulation of actin polymerisation, the formation of transmigratory cups that support leukocyte transmigration and endocytosis of anti-ICAM-1 coated microbeads.[6;13;56;57] Such events may also be involved in the mechanisms through which neutrophil ICAM-1 ligation supports particle engulfment.

Antibody-crosslinking of neutrophil ICAM-1 also enhanced LPS-stimulated intracellular ROS generation. Whilst it is attractive to speculate that this may be associated with pathogen killing within phagocytic vacuoles, it is also potentially possible that the ROS products generated support intracellular signalling. Indeed we observed high levels of ROS signal in cells that had engulfed zymosan (not shown), which may reflect activation of a “pathogen-killing” oxidative burst. In contrast, lower levels of ROS were noted in LPS-stimulated cells, a response that may reflect activation of signalling pathways.

ROS have been intimately associated with numerous signalling pathways triggered by ligation of endothelial cell adhesion molecules, including ICAM-1.[58] Of relevance, ICAM-1 blockade inhibits production of ROS by neutrophils in response to GM-CSF or PMA,[28] and ICAM-1 cross-linking has been linked to ROS generation and signalling in monocytes,[59] indicating a broad role for myeloid cell ICAM-1 signalling as a regulator of superoxide anion

generation. Finally, as neutrophils constitutively express the key ICAM-1 ligands, β2 integrins LFA-1 and Mac-1, ICAM-1-mediated neutrophil adhesion/aggregation may trigger ROS generation in an adhesion-dependent manner, as previously detailed.[60]

The expression and function of neutrophil ICAM-1 was also investigated *in vivo* using murine models of endotoxemia characterised by local neutrophil infiltration and blood neutrophilia. Within these models, tissue infiltrated and blood vascular neutrophils exhibited enhanced ICAM-1 levels. Importantly, IL-1β-induced tissue transmigrated neutrophils showed no ICAM-1 expression indicating that: (i) In line with our *in vitro* findings, *in vivo* neutrophil ICAM-1 induction occurs in a stimulus-specific manner, and (ii) neutrophil transmigration *per se* is not sufficient to elicit ICAM-1 expression on neutrophils. Of relevance, human neutrophils that have migrated through TNF-stimulated cultured endothelial cells exhibit enhanced ICAM-1 expression though the role of this remains unclear.[27] Chimeric mice deficient in myeloid ICAM-1 indicated no involvement for neutrophil ICAM-1 in neutrophil tissue infiltration. In contrast to neutrophils, monocyte ICAM-1 has been shown to mediate adhesion and transendothelial migration, though the associated mechanism remains unknown.[58]

Our chimeric mice did however indicate a significant role for neutrophil ICAM-1 in phagocytosis of zymosan particles *in vivo*. This was evident with respect to both tissue infiltrated and blood neutrophils, responses that could collectively reflect events involved in clearance of both tissue and blood-borne pathogens. With respect to the latter, intravenously injected zymosan particles were most effectively phagocytosed by neutrophils within the pulmonary vasculature. The pulmonary vasculature is composed of an extensive network of small diameter alveolar capillaries through which leukocytes squeeze during normal re-circulation. As a consequence, there is increased transit time of leukocytes through the lungs resulting in a large pool of slow moving marginating leukocytes.[61-64] This low shear environment provides an ideal setting for bringing marginated neutrophils in close and prolonged proximity to blood borne pathogens. Our data suggests that filtration of phagocytic targets from blood, as it passes through the pulmonary vasculature, may be enhanced by neutrophil-expressed ICAM-1. Hence, whilst it is well accepted that lungs are an important host defence organ, this may involve pathogen clearance by phagocytes within the interstitial tissue as well as by circulating neutrophils within the pulmonary vasculature. Of relevance, whilst ICAM-1 deficient mice are protected from a number of sterile inflammatory conditions such as diabetic renal injury, radiation induced lung injury and atherosclerosis,[65-67] they are highly susceptible to models of infections.[68;69] These phenotypes are considered to be largely due to profound inhibition of leukocyte extravasation mediated by lack of endothelial cell ICAM-1. However, our results suggest that neutrophil ICAM-1 deficiency may also

contribute to defective pathogen clearance and hence increased mortality in infectious models. In keeping with these findings is the observation that ICAM-1 KO neutrophils migrate equivalently to wild type neutrophils into *S. pneumoniae* infected lungs, but fail to control the pneumococcal infection.[70] This hypothesis is in line with the fact that elevated neutrophil ICAM-1 has been reported in numerous infectious clinical setting involving bacterial and viral pathogens,[19;20;71] further supporting the concept that elevated neutrophil ICAM-1 is a physiological component of innate immunity.

The mechanism through which neutrophils are retained in the pulmonary vasculature, most notably under conditions of infection, remains an elusive issue. Since fibrinogen can mediate leukocyte-endothelial adhesion,[52] we propose that the interaction of neutrophil ICAM-1 with endothelial cell fibrinogen may provide a mechanism through which neutrophils accumulate in the pulmonary vascular compartment and contribute to luminal pathogen killing and clearance. In support of this, analysis of our chimeric mice indicated a higher ratio of WT:KO neutrophils in the pulmonary vasculature than in the rest of the circulation. Furthermore, fibrinogen deficient mice exhibit delayed pulmonary injury in response to LPS,[72] and whilst this has been linked to endothelial cell-associated fibrinogen binding to neutrophils via P-selectin and integrins, our data suggest that neutrophil ICAM-1 may also contribute to pulmonary retention.

In summary, our data provide insight into regulation of expression and function of neutrophil ICAM-1 *in vitro* and *in vivo*, most notably identifying it as a novel regulator of neutrophil phagocytosis. The findings highlight new avenues of research in host defence and suggest that induced expression of neutrophil ICAM-1 maybe a means through which pathogen clearance maybe enhanced in immunocompromised patients.

Contributions

A.W. & M.B designed and performed most experiments, analysed data and contributed to the writing of the manuscript. M-B.V. designed and performed some immunofluorescent staining experiments. B.M. assisted with image acquisition and analysis. J.R.W. designed and performed RT-PCR experiments. P.H. contributed to the design of some of the experiments and the writing of the manuscript. N.H. provided valuable tools and contributed to the writing of the manuscript. S.N. provided overall project supervision, contributed to the design of experiments and the writing of the manuscript.

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Conflicts of interest

There are no conflicts of interest in this work.

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Figure Legends

Figure 1. **Murine neutrophils exhibit enhanced ICAM-1 expression in a stimulus-specific manner *in vitro*.**

Whole blood from WT or ICAM-1 KO mice was incubated (4 hrs at 37°C) with IL-1β (50 ng/ml), LTB4 (30 nM), KC (30 nM), fMLP (1 μM), Zymosan (10 μg/ml), LPS (300 ng/ml) or TNF (100 ng/ml). Post red blood cell (RBC) lysis step, leukocytes were labelled with DAPI, and fluorescent antibodies against CD45, Ly6G and ICAM-1. Samples were analysed by flow cytometry. (A) Percentage of ICAM-1 positive neutrophils in WT and ICAM-1 KO samples. (B) Relative fluorescent intensity (RFI; as compared to isotype control) of unstimulated and LPS-stimulated WT neutrophils. (C) Representative histogram of ICAM-1 expression on saline or LPS stimulated WT neutrophils, as compared to binding of an isotype control. (D) Time course of cell surface ICAM-1 expression. (E) Naïve or LPS stimulated WT neutrophils labelled with fluorescent antibodies against Ly6G and ICAM-1, and the nuclear marker Draq5. Some samples were permeablised before ICAM-1 labelling to visualise intracellular stores in addition to surface expression. Cells were imaged by confocal microscopy and analysed using Imaris imaging software. (F) Q-PCR analysis of ICAM-1 mRNA in saline or LPS stimulated neutrophils. Unstimulated expression levels were normalised to 1 and LPS stimulated data is shown as fold change compared to unstimulated samples. (G) Western Blot of ICAM-1 and β-actin loading control in naïve or LPS stimulated purified neutrophils treated with protease inhibitor at the time of collection. Data are expressed as mean ± SEM of n=3-27 animals/group. Statistically significant (t-test) differences between treatment groups are indicated by asterisks: *P<0.05, and ***P<0.001, and differences between WT and ICAM-1 KO are indicated by hash symbols: #P<0.05, ##P<0.01 and ###P<0.001

Figure 2. ICAM-1 expression facilitates enhanced neutrophil effector functions.

Whole blood from WT or ICAM-1 KO mice was incubated (4 hrs at 37°C) with IL-1β (50 ng/ml), LTB4 (30 nM), KC (30 nM), fMLP (1 μM), LPS (300 ng/ml) or TNF (100 ng/ml). Fluorescent ZymTR particles (10 μg/ml) or DHR (1 μM) were added to samples for 15 minutes before RBC lysis and labelling with DAPI and fluorescent antibodies against CD45 and Ly6G. Samples were analysed by flow cytometry. (A) Representative histogram illustrating the detection of ZymTR associated neutrophils. (B) Percentage of $ZymTR^{pos}$ neutrophils in unstimulated and LPS-stimulated WT and ICAM-1 KO samples. (C) Percentage of DHR positive neutrophils, in LPS stimulated as compared to unstimulated controls was quantified in WT and ICAM-1 KO samples. (D) Frequency of DHR positive neutrophils within the ICAM-1 positive or negative populations in LPS-stimulated blood. Data are expressed as mean ± SEM of n=4-24 animals/group. (E) Percentage of DHR positive neutrophils in WT neutrophils stimulated as shown. Statistically significant (t-test) differences between treatment groups are indicated by asterisks: **P<0.01, and ***P<0.001, and differences between WT and ICAM-1 KO are indicated by hash symbols: #P<0.05, ###P<0.001.

Figure 3. **Mechanism of neutrophil ICAM-1-mediated enhanced effector functions.**

Whole blood from WT or ICAM-1 KO mice was incubated with LPS (300 ng/ml, 4 hrs at 37℃). In some experiments, samples were treated with inhibitors as detailed, prior to addition of ZymTR for a further 15 minutes. Unless indicated, results are presented as percentage inhibition of LPS-stimulated ZymTR phagocytosis. (A) LPS-stimulated samples were treated with blocking antibodies against ICAM-1 (clone YN1/1.7.4) or Mac-1 (clone M1/70) (both at 10 µg/ml for 15 minutes). (B) LPS-stimulated samples were treated with fibrinogen-γ-117-113 peptide to block ICAM-1-fibrinogen interaction (300 µM for 30 minutes). (C) LPS-stimulated samples were incubated with a membrane penetrating peptide consisting of 13 C-terminal amino acids of ICAM-1 that inhibits ICAM-1-mediated intracellular signalling (200 µg/ml for 120 minutes at 37℃). (D) LPS-stimulated WT or ICAM-1 KO samples were treated with the Syk inhibitor Piceatannol (30 minutes). (E) Percentage of DHR positive neutrophils in saline or LPS-stimulated samples following antibody cross-linking of ICAM-1 using rat-anti-ICAM-1 primary antibody (10 µg/ml, 15 minutes) and anti-rat secondary antibody (10 µg/ml, 15 minutes). Data are expressed as mean ± SEM of n=8-15 animals/group. Statistically significant (t-test) differences between control and treatment groups are indicated by asterisks: *P<0.05, **P<0.01, and ***P<0.001, and differences between WT and ICAM-1 KO treatments are indicated by hash symbols: ###P<0.001.

Figure 4. ICAM-1 is expressed on LPS-stimulated neutrophils *in vivo*.

WT mice were injected with saline, LPS (300 ng intrascrotal route [i.s]. or 1000 ng intraperitoneal route [i.p.]) or IL-1β (50 ng i.p and i.s.). After a 4 hour in vivo test period, blood, lungs, peritoneal lavage and cremaster muscles were collected. Cremaster muscles were fixed and permeablised and labelled with fluorescent antibodies against PECAM-1, the intracellular neutrophil marker S100a9 and ICAM-1 or an isotype control. Tissues were imaged by confocal microscopy and analysed using Imaris. Following RBC lysis and enzymatic digestion of lung tissues, leukocytes from the blood, lung tissue digest and peritoneal lavage were labelled with DAPI and fluorescent antibodies against CD45, Ly6G and ICAM-1, or an isotype control. (A) Representative images of unstimulated and stimulated cremaster muscles. (B and C) Quantification of extravasated neutrophils in saline and stimulated inflamed sites. (D) Representative images of ICAM-1 expression on extravasated neutrophils in LPS- and IL-β-stimulated cremaster muscles. (E and F) Quantification of ICAM-1 expression on extravasated neutrophils in inflamed tissues. (G) Percentage of Ly6G positive neutrophils amongst CD45 positive leukocytes in blood and digested lung tissue samples as quantified by flow cytometry. (H) Percentage of ICAM-1 positive neutrophils in blood of saline, IL-1β- and LPS-stimulated animals. Data are expressed as mean ± SEM of n=3-10 animals/group. Statistically significant (t-test or ANOVA) differences between stimulated and unstimulated treatment groups are indicated by asterisks: *P<0.05, **P<0.01, ***P<0.001. Differences between stimuli are indicated by hash symbols: ##P<0.01, ###P<0.001.

Figure 5. Neutrophil ICAM-1 does not support neutrophil transmigration *in vivo*.

Extravasation of WT or ICAM-1 KO neutrophils was quantified using chimeric WT mice with a mix of WT-LysM-GFP and ICAM-1 KO (GFP negative) neutrophils. Chimeric mice were stimulated with LPS (1000 ng i.s. or i.p.) and cremaster muscle tissues and peritoneal lavage fluid was collected and labelled with fluorescent antibodies against PECAM-1 and S100a9 (cremasters), or CD45 and Ly6G (lavage). (A) Representative image of extravasated WT

and ICAM-1 KO neutrophils in LPS-stimulated cremaster muscles. (B and C) Quantification of extravasated WT-LysM-GFP and ICAM-1 KO neutrophils in saline and/or LPS-stimulated inflamed tissues. Values are normalised to the WT:KO ratio in the peripheral blood of each animal. Data are expressed as mean ± SEM of n=6-10 animals/group. Statistically significant (t-test or ANOVA) differences between stimulated and unstimulated treatment groups are indicated by asterisks: *P<0.05, **P<0.01.

Figure 6. ICAM-1 facilitates neutrophil phagocytosis *in vivo*.

Chimeric WT mice with a mix of WT-LysM-GFP and ICAM-1 KO (GFP negative) neutrophils were injected with LPS (1000 ng i.p.). After 4 hrs ZymTR was injected i.p. (10 µg) or i.v. (100 µg) 15 minutes before collection of blood, lung tissues and peritoneal lavage. Following RBC lysis and enzymatic digestion of lung tissues, cells were labelled with DAPI and fluorescent antibodies against CD45 and Ly6G, and analysed by flow cytometry. (A) Percentage of ZymTR associated WT-LysM-GFP or ICAM-1 KO neutrophils in peritoneal lavage. (B) Percentage of ZymTR associated WT-LysM-GFP or ICAM-1 KO neutrophils in blood and enzymatically digested lung tissues. (C) Total number of $ZymTR^{pos}$ WT-LysM-GFP or ICAM-1 KO neutrophils in the lungs of saline or LPS stimulated animals. Values are normalised to the WT:KO ratio in the tissue of each animal. (D) Representative images showing neutrophils and ZymTR particles in LPS stimulated pulmonary or cremasteric circulation 15 minutes after i.v. injection of ZymTR. Data are expressed as mean ± SEM of n=6-10 animals/group. Statistically significant (t-test) differences between treatment groups are indicated by asterisks: *P<0.05. Differences between WT-LysM-GFP and ICAM-1 KO are indicated by hash symbols: ##P<0.01, ###P<0.001.

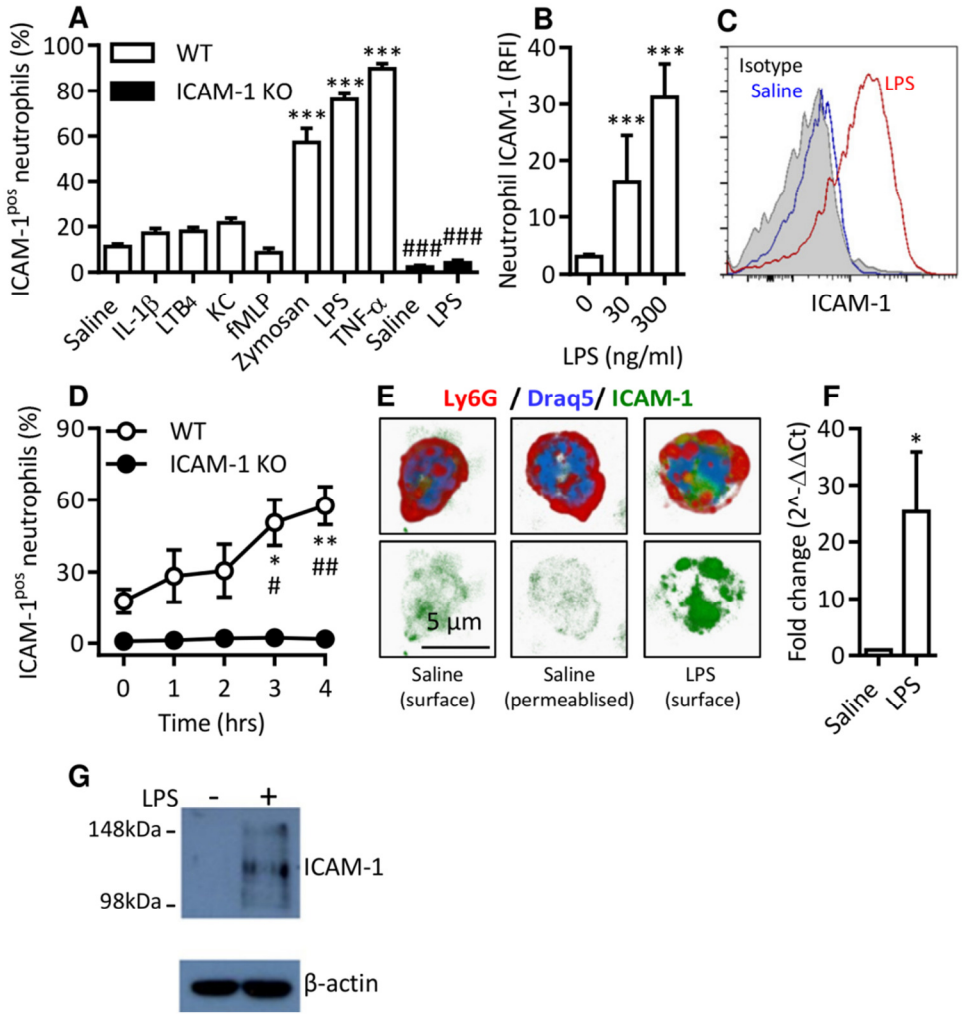

A
ICAM-1pos neutrophils (%)
WT
ICAM-1 KO
Saline
IL-1β
LTB4
KC
fMLP
Zymosan
LPS
TNF-α
Saline
LPS
B
Neutrophil ICAM-1 (RFI)
LPS (ng/ml)
C
Isotype
Saline
LPS
ICAM-1
D
ICAM-1pos neutrophils (%)
WT
ICAM-1 KO
Time (hrs)
E
Ly6G / Draq5/ ICAM-1
5 µm
Saline (surface)
Saline (permeablised)
LPS (surface)
F
Fold change (2^-ΔΔCt)
Saline
LPS
G
LPS
148kDa
98kDa
ICAM-1
β-actin

Figure 1

1

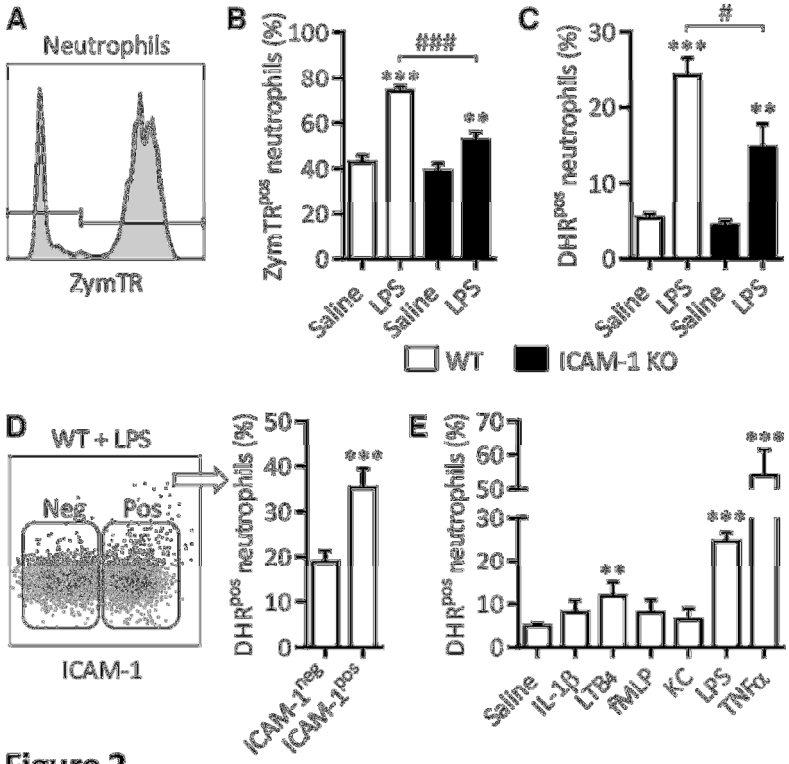
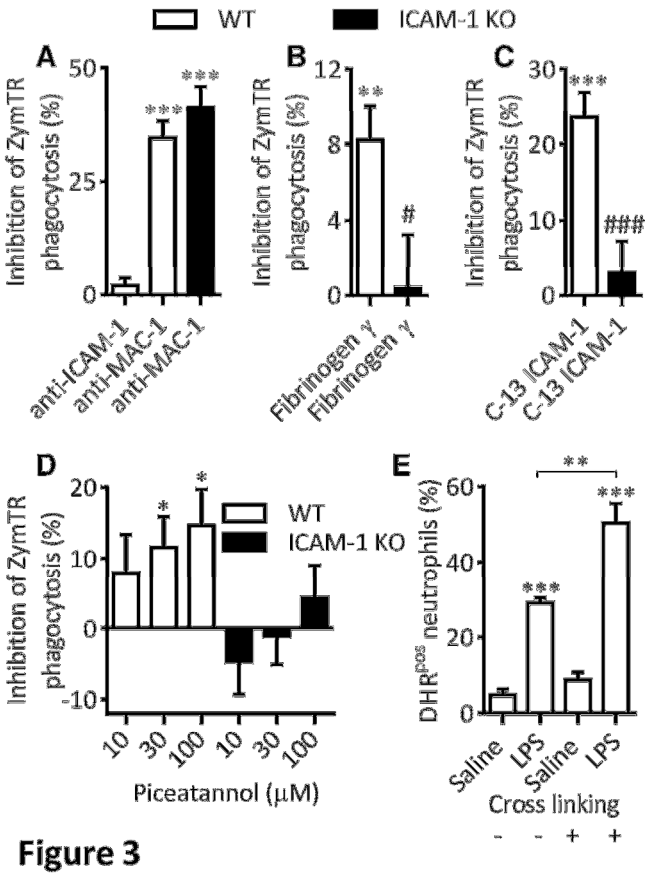

A
Neutrophils
ZymTR
B
ZymTR^pos neutrophils (%)
###
***
**
Saline
LPS
Saline
LPS
C
DHR^pos neutrophils (%)
#
***
**
Saline
LPS
Saline
LPS
WT
ICAM-1 KO
D
WT + LPS
Neg
Pos
ICAM-1
DHR^pos neutrophils (%)
***
ICAM-1^neg
ICAM-1^pos
E
DHR^pos neutrophils (%)
**
***
***
Saline
IL-1β
LTB4
fMLP
KC
LPS
TNFα

Figure 2

1


WT
ICAM-1 KO
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Inhibition of ZymTR phagocytosis (%)
anti-ICAM-1
anti-MAC-1
anti-MAC-1
B
Inhibition of ZymTR phagocytosis (%)
Fibrinogen γ
Fibrinogen γ
C
Inhibition of ZymTR phagocytosis (%)
C-13 ICAM-1
C-13 ICAM-1
D
Inhibition of ZymTR phagocytosis (%)
WT
ICAM-1 KO
Piceatannol (μM)
E
DHR^pos neutrophils (%)
Saline
LPS
Saline
LPS
Cross linking
- - + +

Figure 3

1

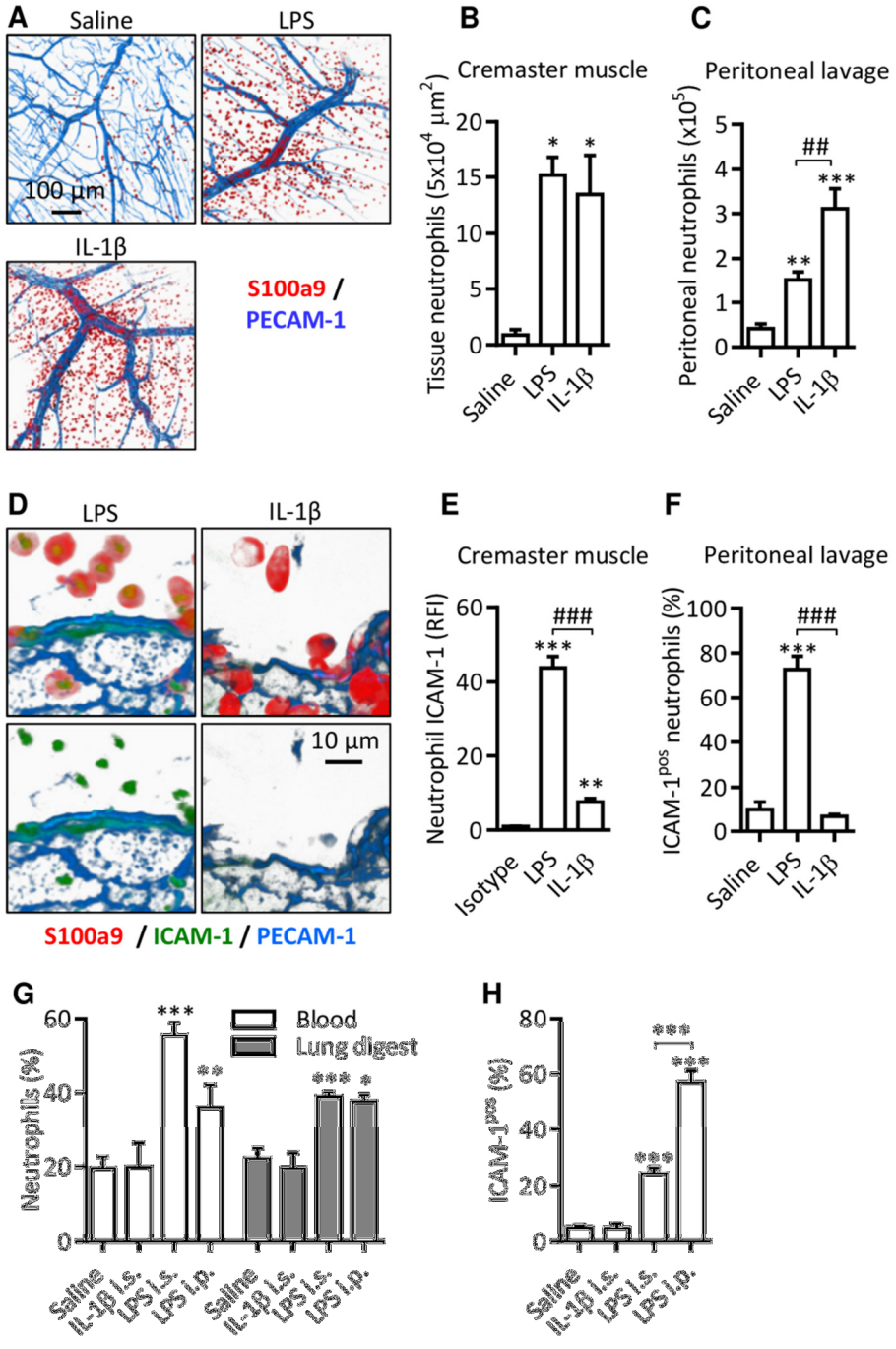
A
Saline
LPS
100 µm
IL-1β
S100a9 /
PECAM-1
B
Cremaster muscle
Tissue neutrophils (5x10^4 µm^2)
Saline
LPS
IL-1β
C
Peritoneal lavage
Peritoneal neutrophils (x10^5)
D
LPS
IL-1β
10 µm
S100a9 / ICAM-1 / PECAM-1
E
Cremaster muscle
Neutrophil ICAM-1 (RFI)
Isotype
F
Peritoneal lavage
ICAM-1pos neutrophils (%)
G
Blood
Lung digest
Neutrophils (%)
Saline
IL-1β i.s.
LPS i.s.
LPS i.p.
H
ICAM-1pos (%)

Figure 4

1

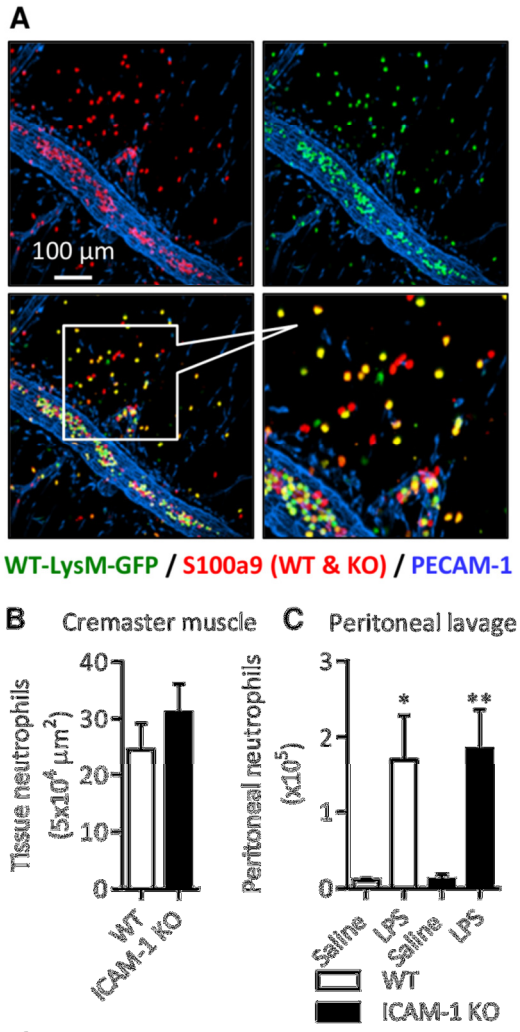
A
100 µm
WT-LysM-GFP / S100a9 (WT & KO) / PECAM-1
B
Cremaster muscle
Tissue neutrophils
(5x10⁴ µm²)
40
30
20
10
0
WT
ICAM-1 KO
C
Peritoneal lavage
Peritoneal neutrophils
(x10⁵)
3
2
1
0
*
**
Saline
LPS
Saline
LPS
WT
ICAM-1 KO

Figure 5

1

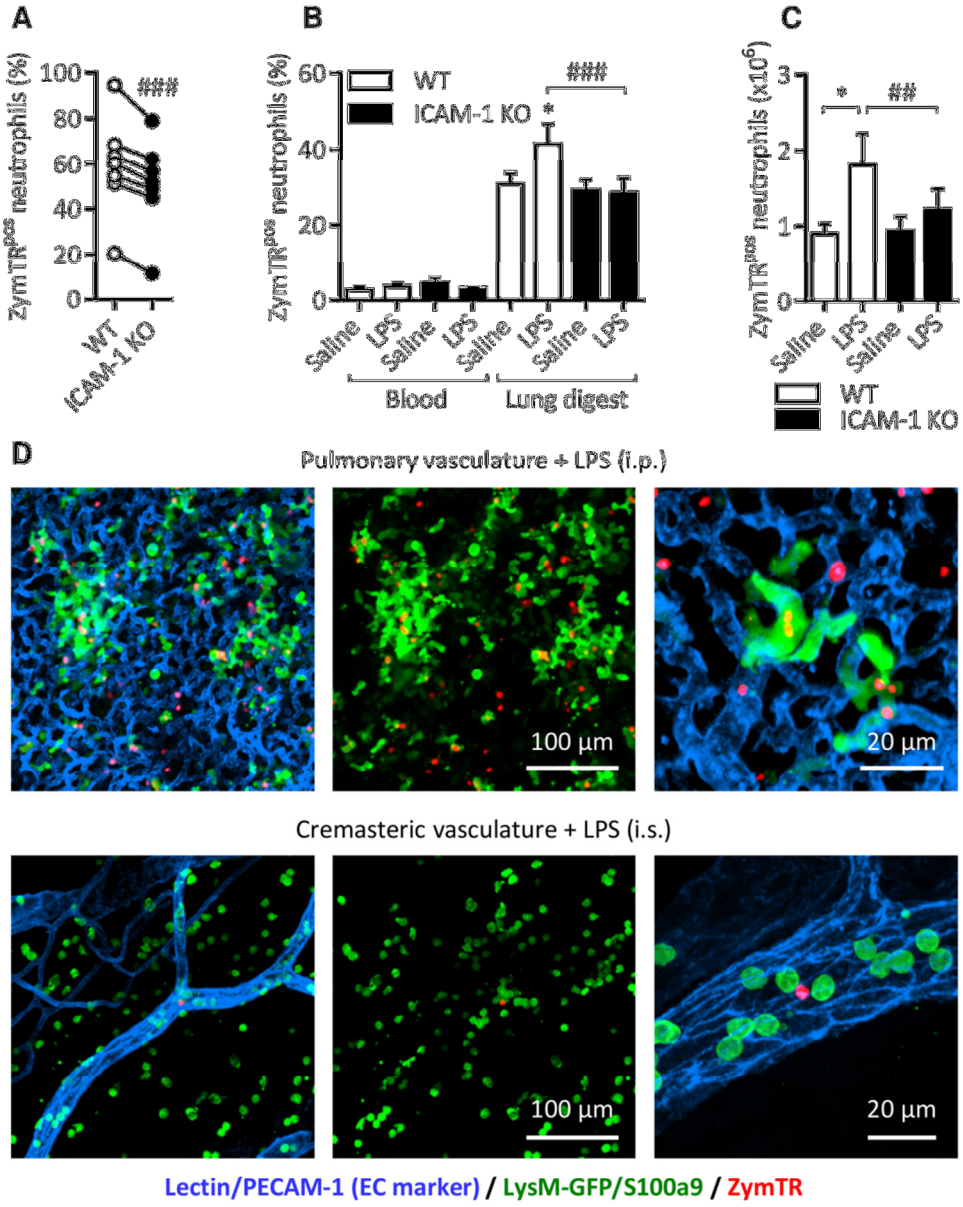
A
B
C
D
ZymTR^pos neutrophils (%)
ZymTR^pos neutrophils (x10^6)
###
WT
ICAM-1 KO
Saline
LPS
Blood
Lung digest
*
##
Pulmonary vasculature + LPS (i.p.)
Cremasteric vasculature + LPS (i.s.)
100 µm
20 µm
Lectin/PECAM-1 (EC marker) / LysM-GFP/S100a9 / ZymTR

Figure 6

1

Supplementary Materials and Methods

Animals

Male WT C57BL/6 mice were purchased from Charles River Laboratories. ICAM-1 KO animals were obtained from Drs Britta Engelhardt and Urban Deutsch with permission of Dr Art Beaudet[1]. Heterozygous mice in which the gene for eGFP has been knocked-in to the lysozyme M (LysM) locus (*LysM-eGFP-ki*),[2] resulting in the exhibition of eGFP high levels of eGFP in neutrophils and to a lesser extent monocytes were used to distinguish WT and ICAM-1 KO PMN in mixed populations. These mice are now referred to as WT-LysM-eGFP to indicate that they contain functional ICAM-1 genes. All genetically modified animals were on a C57BL/6 background. Animals were housed in individually ventilated cages and facilities were regularly monitored for health status and infections. Experiments were performed under the UK legislation for the protection of animals, and at the end of all *in vivo* procedures animals were humanely killed by cervical dislocation in accordance with UK Home Office regulations.

Reagents & antibodies

IL-1β, LPS, LTB_4, fMLP, DAPI and EZ-view Protein G affinity gel were purchased from Sigma-Aldrich. Dihydrorhodamine-123 (DHR), AlexaFluor antibody conjugation kits and Zymosan-Texas-Red (ZymTR) were purchased from Invitrogen. Antibodies were purchased from eBioscience, Biolegend, Abcam, Pharmingen, Santa Cruz, Sigma-Aldrich or were provided by Research Cell Services, Cancer Research UK London Research Institute: anti-CD45 (clone 30-F11), anti-Ly6G (clone 1A8), anti-ICAM-1 (clone YN1/1.7.4), anti-ICAM-1 (clone H-108), anti-β-actin (cloneAC-74), anti-PECAM-1 (clone C390), anti-MAC-1 (clone M1/70), anti-S100a9 (clone 2B10), rat IgG2b and IgG2a isotype controls. For confocal imaging or flow cytometry primary antibodies were either directly conjugated commercially (PE, FITC, PECy7, Pacific Blue, Alexa-488, Alexa-555, Alexa-633, Alexa-700) or labelled with Invitrogen AlexaFluor antibody conjugation kits in house. ICAM-1/fibrinogen binding inhibitor peptide was synthesised by Bachem. ICAM-1 signalling inhibitor peptide was synthesized by Cambridge Peptides. Qiagen RNeasy Microkit iScript cDNA synthesis kit and Sybr green supermix were purchased from (Biorad). ICAM-1 and GAPDH primers were purchased from Integrated DNA Technologies. HALT™ protease and phosphotase inhibitor cocktail was purchased from Pierce.

Generation of chimeric mice

Generation bone marrow chimeric mice was based on published methods [3]. Bone marrow was isolated from the tibias of WT, ICAM-1 KO or WT-LysM-eGFP mice under sterile conditions and suspended in sterile PBS at $5x10^6$/ml. WT recipient mice were lethally irradiated with two doses of 5 Gy, 4 hours apart using a RadSource-2000 irradiator. After the second irradiation mice were given an intravenous (i.v.) injection of $1x10^6$ bone marrow cells in 200 µl PBS. After irradiation and bone marrow transfer animals were maintained in individually ventilated cages and given acidified (pH 2.6) water and Baytril for 4-6 weeks before use. Chimeric mice had an average ratio of 60:40 WT:KO neutrophils in the circulation, and data from each animal was normalised to the individual ratio (see also Supplementary Figure 1).

In vivo inflammatory responses

Animals were sedated via intramuscular (i.m.) injection of 1 ml/kg anaesthetic (40 mg/kg Ketamine and 2 mg/kg Xylazine in saline) and given intrascrotal (300 µl i.s.) or intraperitoneal (500 µl i.p.) injections of LPS (300ng or 1000 ng) or IL-1β (50 ng), with an incubation period of 4 hrs. In some cases fluorescent Zymosan-Texas-Red (ZymTR) was given i.p. (10 µg) or i.v. (100 µg) 15 minutes prior to collection of blood, lungs, peritoneal lavage or cremasters. In some cases i.s. injections of inflammatory mediators were co-administered with fluorescent anti-PECAM-1 mAb (4 µg) to label the cremasteric vasculature. Animals were sedated (125 mg/kg Ketamine, 12.5 mg/kg Xylazine) i.p. or killed by cervical dislocation. Blood was collected into heparinised PBS via cardiac puncture or cannulation of the abdominal vena cava. To collect the lungs the chest was opened and a clamp was placed on the thoracic vena cava and aorta just above the diaphragm. The pulmonary vasculature of the lungs was then perfused with 10 ml of PBS containing heparin and EDTA by direct injection into the right ventricle, and collection via a cannula inserted into the left ventricle. Vascular wash out was confirmed by whitening of the lung tissue, which was then excised, finely chopped, and digested for 30 minutes in 1 ml PBS with collagenase and DNAse (500 U and 200 U respectively), and passed through a 40 µm cell sieve. The peritoneal cavity was lavaged with 6 ml PBS/EDTA and cells we counted using a haemocytometer and analysed by flow cytometry to assess extravasation and phagocytosis efficiency. Cremasters were also collected, fixed, permeablised and immunofluorescently labelled.

***In vitro* stimulation**

Neutrophil responses to various *in vitro* inflammatory stimuli were analysed. Whole blood was collected into heparinised PBS from naïve mice via cannulation of the vena cava and treated with LPS (30 or 300 ng/ml), IL-1β (100 ng/ml), LTB_4 (30 nM), KC (30 nM) or fMLP (1 µM), TNFα (100 ng/ml) or Zymosan (10 µg/ml) for up to 4 hrs at 37˚C. Phagocytosis was quantified by addition of 10 µg/ml fluorescent ZymTR to whole or lysed blood, and incubation for 15 minutes at 37˚C (approximate ratio of 10:1 ZymTR:neutrophils). As has been previously reported[4] blood from naïve ICAM-1 KO mice exhibited significantly elevated numbers of neutrophils (WT 0.53±0.06, ICAM-1 KO 0.73±0.07 million/ml, $P<0.05$*), but the high ratio of ZymTR:neutrophils would negate this difference. ROS generation was quantified using the fluorescent intracellular probe DHR added to whole blood 15 minutes prior to lysis and analysis (1 µM). In some cases function blocking antibodies or the inhibitor Piceatannol was added prior to ZymTR. In some cases a 17 amino acid peptide from the gamma domain of fibrinogen that binds to domain 1 of ICAM-1 and competitively inhibits ICAM-1/fibrinogen interactions was used (sequence: NNQKIVNLKEKVAQLEA).[5] Whole blood was stimulated with 300 ng/ml LPS for 3 hrs before addition of 300 µM peptide for a further 30 minutes, and ZymTR (10 µg/ml) for a further 15 minutes before lysis and analysis by flow cytometry. In some cases a biotinylated peptide comprised of a 16 amino acid cell membrane penetrating sequence followed by 13 C-terminal amino acids of mouse ICAM-1 was used to inhibit intracellular signalling of ICAM-1: (sequence Bio-RQIKIWFQNRRMKWKK-QRKIRIYKLQKAQ).[6] Whole blood was stimulated with 300 ng/ml LPS for 3 hrs before addition of 200 µg/ml inhibitor for a further 2 hrs, and ZymTR (10 µg/ml) for a further 15 minutes before lysis and analysis by flow cytometry.

Flow Cytometry

Whole blood from *in vitro* stimulations, or whole blood, peritoneal lavage or digested lung tissue from *in vivo* experiments, were lysed with ACK lysis buffer as required (150 mM

NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA), and stained with fluorescently conjugated antibodies against CD45, Ly6G, ICAM-1, CD18, or the appropriate isotype control antibodies (0.2 – 2 µg/ml, various fluorochromes). Leukocytes were identified by FSC and SSC characteristics and CD45 positive staining, dead cells were excluded by DAPI staining, and neutrophils were identified based on Ly6G mAb staining or LysM-eGFP positivity. ICAM-1 was labelled with an APC conjugated antibody and positive neutrophils were gated after determining the level of background fluorescence in the ICAM-1 KO or isotype control stained samples using 633/670 nm excitation/emission. ZymTR associated neutrophils from *in vitro* or *in vivo* experiments were identified by 560/585 nm excitation/emission. ROS generation was quantified by loading cells with 1 µM DHR for 15 minutes at 37˚C, DHR fluorescence was quantified by flow cytometry at 488/530 nm excitation/emission. DHR positive neutrophils were identified as cells with a higher DHR signal than an unstimulated, but DHR loaded, control. Samples were analysed using a BD LSR-Fortessa (BD Biosciences) and FloJo analysis software (Treestar). In some studies the intracellular or surface bound location of neutrophil associated ZymTR was analysed using the ImageStream^X Mk II imaging cytometer, which combines fluorescence microscopy with high-throughput flow cytometry. Neutrophils were identified as being positive for CD45 and Ly6G labelling, and a mask was applied to the Ly6G positive regions of ZymTR positive neutrophil events. The frequency at which the ZymTR signal fell within the Ly6G mask (intracellular) or outside the Ly6G mask (surface bound) was quantified.

Confocal microscopy

Cremasters were fixed with 4% PFA, blocked with 20 % FCS and permeablised with 0.5 % Triton-X-100. Cremasters were incubated with fluorescent anti-PECAM-1, anti-S100a9, anti-ICAM-1 or isotype control. Lung tissues from WT-LysM-GFP mice were incubated with 5 µg/ml Lectin IB4-Alexa-647 for 2 hours before the surface of the lungs was imaged. Tissues, or isolated neutrophils, were viewed using a Leica SP5 confocal microscope incorporating a 20× water-dipping objective (NA 1.0). For analysis of LPS and IL-1β stimulated extravasation *Z*-stack images of 3-5 post-capillary venules with 20–45 µm diameters per cremaster were captured, and extravasated neutrophils were quantified (3-4 mice per group). ICAM-1 expression on extravasated neutrophils was carried out by building an isosurfaces on the S100a9 channel, and quantifying the ICAM-1 signal within this surface. Image analysis carried out using Imaris (Bitplane) 3D analysis software or Leica LASF-Lite software.

Quantitative RT-PCR

Neutrophils were purified from blood or lung digests by flow sorting for analysis of ICAM-1 mRNA levels using BD FACS-ARIA (BD Biosciences). Leukocytes were identified by FSC and SSC characteristics and CD45 positive staining, dead cells were excluded by DAPI staining, and neutrophils were identified based on Ly6G positivity. Purified samples were 84.9 % neutrophils. Following purification total RNA was purified from cells using the Qiagen RNeasy Microkit as described by the manufacturers. cDNA was synthesized from 10 ng total RNA using the iScript cDNA synthesis kit. Quantitative Real Time PCR was performed on an ABI7900HT real time PCR machine and reactions were prepared using the iQ Sybr green supermix.

ICAM - Forwards: GACAGTACTGTACCACTCTC, Reverse:CCTGAGCCTTCTGTAACTTG,

GAPDH - Forwards:TCGTGGATCTGACGTGCCGCCTG, Reverse:CACCACCCTGTTGCTGTAGCCGTA.

Western Blot

WT blood was stimulated with 1000 ng/ml LPS or saline for 4 hours. Ly6G^{pos} Neutrophils were purified from blood or lung digests by flow sorting using BD FACS-ARIA (BD Biosciences), and pellets re-suspended in RIPA buffer with Halt^{TM} protease inhibitor cocktail. Cells were run on a 10% gel and transferred by semi-dry transfer. ICAM-1 expression and β-actin loading control were detected by anti-ICAM-1 (1:50, clone H-108), anti-β-actin (1:2000, clone AC-74).

Statistical analysis

Results are presented as mean ± s.e.m. Statistical significance was assessed by the Student T-test or by one-way analysis of variance (ANOVA) with the Newman-Keuls multiple comparison test. Where T-tests were used a paired T-test was used to compare samples from the same animal which had been subjected to different treatments (e.g. unstimulated and LPS stimulated blood), while comparison of samples from different animals were analysed using an unpaired test. P values below 0.05 were considered significant.

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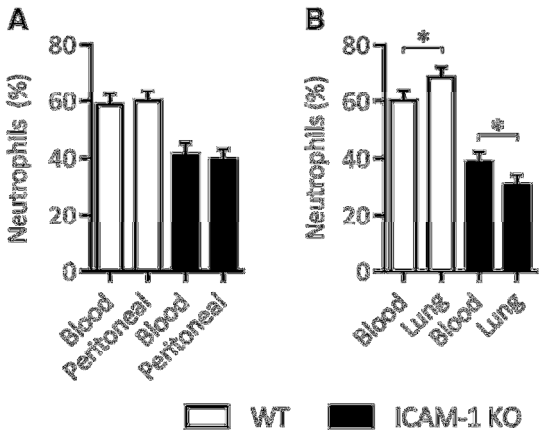

Supplementary Figure 1. WT and ICAM-1 KO neutrophils in chimeric mice.

A mixture of WT-LysM-eGFP and ICAM-1 KO bone marrow was injected intravenously (i.v.) to irradiated WT mice to create chimeric mice that expressed ICAM-1 on the vasculature with ICAM-1 KO or WT-LysM-GFP leukocytes. (A) There was a 60:40 WT:ICAM-1 KO neutrophils in the blood. (B) In digested lung tissues there was a slight increase in the proportion of WT neutrophils as compared to the blood (from 60.1±3.8% to 68.7±3.6%), indicating a possible preferential retention of WT cells in the vasculature of the lung. Data are expressed as mean ± SEM of n=8-12 animals/group. Statistically significant (t-test) differences are indicated by asterisks: *P<0.05.