

## **BRIEF CONCLUSIVE REPORT**

### **Regulation of ICAM-1 in human neutrophils**

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## **ABSTRACT**

Intercellular cell adhesion molecule 1 (ICAM-1) is a cell surface glycoprotein with a vital role in the immune response to pathogens. The expression pattern of ICAM-1 is wide-ranging, encompassing endothelial cells, epithelial cells and neutrophils. Recent work has characterized the role of ICAM-1 in murine neutrophils, but the function of human neutrophil ICAM-1 is incompletely understood. Herein, we investigated the expression and role of ICAMs in human neutrophils *in vitro* and *in vivo*. Our findings show clear expression of ICAM-1, -3 and -4 on peripheral blood-derived neutrophils and demonstrate that the pathogen-associated molecular pattern (PAMP) lipoteichoic acid (LTA) is an inducer of ICAM-1 expression *in vitro*. *In vivo*, neutrophils obtained from the pleural cavity of patients with a parapneumonic effusion display enhanced expression of ICAM-1 compared to peripheral blood- and oral cavity-derived neutrophils. Moreover, migration of peripheral blood-derived neutrophils across endothelial cells can upregulate neutrophil ICAM-1 expression. These findings indicate that PAMPs and/or cytokines, alongside transmigration, enhance neutrophil ICAM-1 expression at sites of inflammation. Mechanistically we observed that ICAM-1<sup>high</sup> neutrophils display elevated *S. aureus* phagocytic capacity. However, unlike murine neutrophils, ICAM-1 intracellular signaling in human neutrophils was not essential for phagocytosis of *S. aureus* and reactive oxygen species (ROS) generation. Taken together, these results have important implications for the regulation of neutrophil-mediated pathogen clearance.

## INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1) is a glycoprotein and member of the Ig superfamily of adhesion proteins, which includes the members ICAM-1 to ICAM-5.<sup>1</sup> ICAM-1 is expressed on the surface of several cell types including endothelial, epithelial and immune cells.<sup>2</sup> On endothelial cells, ICAM-1 has a well-established role mediating leukocyte-endothelial interactions; it regulates leukocyte rolling and adhesion by interacting with lymphocyte function-associated antigen-1 (LFA-1) and leukocyte macrophage antigen 1 (Mac-1).<sup>3,4</sup> Although expressed at low levels under basal conditions, ICAM-1 expression in endothelial cells is up-regulated in response to inflammatory mediators, including lipopolysaccharide (LPS) and tumour necrosis factor (TNF).<sup>5-7</sup> These leukocyte-endothelial interactions are key regulators of leukocyte trafficking at sites of inflammation.

Recently, the importance of ICAM-1 expressed by immune cells has emerged. Studies have shown that dendritic cells and natural killer cells use ICAM-1 for T cell binding and the formation of immune synapses.<sup>8,9</sup> In T cells, ICAM-1 expression supports T cell activation and B cell interactions.<sup>10-12</sup> The expression of ICAM-1 can be induced in inflammatory macrophages, where it can facilitate apoptotic cell clearance.<sup>13</sup> ICAM-1 is also expressed on neutrophils, and can be up-regulated in response to a range of inflammatory stimuli including lipopolysaccharide (LPS), TNF and *S. aureus*.<sup>14,15</sup> Of clinical relevance is the enhanced expression of neutrophil ICAM-1 in patients with peritonitis, sepsis and respiratory syncytial virus (RSV) bronchiolitis,<sup>16-20</sup> highlighting a potential role for neutrophil ICAM-1 in response to pathogens.

Whilst previous work has identified a role for ICAM-1 in neutrophil homotypic aggregation,<sup>14</sup> a detailed characterization of human neutrophil ICAM expression and its role in pathogen clearance is lacking. Such investigations are timely, given the development of ICAM-1 targeted drugs,<sup>21,22</sup> including a recent lung-targeted approach.<sup>23</sup> This study was therefore conducted to examine the expression patterns of human neutrophil ICAM-1 *in vitro* and *in vivo*, as well as the role of ICAM-1 in phagocytosis of *S. aureus*, a pathogen responsible for severe respiratory infections.

## **2. METHODS**

### **2.1 Study participants**

Human peripheral neutrophils were isolated from healthy volunteers. The subjects all gave written informed consent and the study was approved by the Cambridgeshire 2 Research Ethics Committee (08/H03306/17) and North West - Greater Manchester Central Research Ethics Committee (22/NW/0028).

### **2.2 Isolation of blood-derived neutrophils**

Blood-derived neutrophils were isolated from sodium citrate anticoagulated peripheral venous blood, using discontinuous plasma-Percoll gradients, as previously described.<sup>24,25</sup>

### **2.3 Flow cytometry**

Blood-derived neutrophils were resuspended at  $5 \times 10^6$ /ml in RPMI-1640 (Gibco-ThermoFisher, Paisley, UK) with 1% autologous serum and incubated at 37°C (5% CO<sub>2</sub>). Cells were incubated with the following (or vehicle control): Ultra pure LPS-EB (Source BioScience, Nottingham, UK), lipoteichoic acid (BioRad, Hercules, CA, USA), TNF, IL-6,

and IL-8 (R&D Systems, Minneapolis, MN, USA). Ultra-pure LPS-EB was used to ensure removal of contaminating lipoproteins. Neutrophils were stained with anti-ICAM-1-APC (BD Biosciences, San Jose, CA) or IgG1 $\kappa$  isotype control-APC (BD Biosciences) before measurement using flow cytometry (Canto II, BD Biosciences) and analysis using FlowJo™ (Tree Star, Ashland, OR, USA). Data show delta mean fluorescence intensity (MFI), calculated as follows: delta MFI = test - isotype control, unless otherwise indicated.

#### **2.4 Neutrophil transmigration across endothelial cell coated transwells**

Human umbilical vein endothelial cells (HUVECs;  $1.5 \times 10^5$  cells per insert) were added to 3  $\mu$ m pore Transwell polycarbonate inserts (Corning). Once confluent, media was changed and blood-derived neutrophils ( $2 \times 10^6$ /ml) added to the upper chamber in media containing 0.15% BSA and IL-8 (10 ng/ml) to the lower chamber. In control wells, neutrophils were incubated without transwell inserts at  $2 \times 10^6$ /ml in media containing 0.15% BSA. After 2 hours, transwell inserts were removed and the transmigrated neutrophils in the lower chamber counted and resuspended at  $5 \times 10^6$ /ml. Neutrophils were stained with anti-ICAM-1-APC (BD Biosciences, San Jose, CA) or IgG1 $\kappa$  isotype control-APC (BD Biosciences) before measurement using flow cytometry (Canto II, BD Biosciences) and analysis using FlowJo™ (Tree Star, Ashland, OR, USA). Data show delta mean fluorescence intensity (MFI), calculated as follows: delta MFI = test - isotype control, unless otherwise indicated.

#### **2.5 Isolation of oral-cavity-derived neutrophils**

Mouth rinses were collected from subjects by washing with 5 ml of 0.9% saline for 30 sec (for a total of 6 washes). Samples were sequentially filtered through 40 and 20  $\mu$ m meshes (Millipore, Billerica, MA, USA) before centrifugation at 500g for 10 min at RT. Cells were

stained with anti-ICAM-1-APC and neutrophils identified by flow cytometry based on their FSC/SSC and CD16 expression (anti-CD16-FITC).

## **2.6 Isolation of pleural-derived neutrophils**

Pleural fluid samples were centrifuged at 256g for 5 min at RT to obtain a cell pellet. Cell-free supernatants were obtained by centrifugation at 1500g for 15 min and stored at -70°C. Cells were stained with anti-ICAM-1-APC and anti-CD16-FITC (Clone 3G8, BioLegend, San Diego, CA), as described above.

## **2.7 Cytokine measurements**

Cytokines (IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IFN $\gamma$ , and TNF) in cell-free pleural fluid were measured using a 10-plex human cytokine assay (Meso Scale Discovery, Rockville, Maryland, USA). Measurements were performed according to manufacturer's instructions.

## **2.8 Assessment of whole blood neutrophil phagocytosis**

Peripheral venous blood, anticoagulated in argatroban (150  $\mu$ g/ml, R&D Systems), was stimulated with LPS (100 ng/ml) or vehicle control. All conditions were kept on a thermomixer (Invitrogen-Thermofisher, Carlsbad, CA, USA) at 300 rpm and 37°C before incubation with 20  $\mu$ g/ml pHrodo<sup>TM</sup> red *S. aureus* BioParticles (Invitrogen-Thermofisher) for 30 min at 37°C (5% CO<sub>2</sub>). Cells were stained with anti-ICAM-1 BV421 (Clone HA58, BD Biosciences) and anti-CD16-FITC and analysed using an Attune NxT Acoustic Focusing Cytometer (Invitrogen-Thermofisher). Phagocytic index (PI) was determined using FlowJo<sup>TM</sup> and calculated as the % pHrodo<sup>TM</sup> red positive cells  $\times$  pHrodo<sup>TM</sup> red median fluorescence intensity, as previously described.<sup>26</sup>

For experiments using the ICAM-1 blocking peptide, a penetratin linked ICAM-1 C terminal peptide (RQIKIWFQNRRMKWKKQRKIKKYRLQQAQ; Peptide 2.0, Chantilly, VA, USA) or scrambled peptide (RQIKIWFQNRRMKWKKVDDSDDFESVVSV; Peptide 2.0) was used.

## **2.9 Statistical analysis**

Data are presented as median and interquartile range (IQR) or mean and standard deviation (SD) according to whether data are normally distributed. Parametric or non-parametric statistical tests were applied as appropriate after data were tested for normality using the Kolmogorov-Smirnov test. Statistical analysis was performed by using Prism V6.0f software (GraphPad, La Jolla, CA, USA).

## **3. RESULTS AND DISCUSSION**

### **ICAM expression and regulation in human neutrophils**

To characterise the regulation of ICAM-1 we first assessed the basal and induced expression of ICAM-1 in human neutrophils using LPS and lipoteichoic acid (LTA). Peripheral blood derived neutrophils were stimulated for 6 h at 37°C with LPS or LTA, and ICAM-1 expression assessed by flow cytometry (Fig 1A and B). Addition of LPS increased ICAM-1 expression from 1092 MFI (877 – 1321) (Control) to 2582 MFI (2233 – 3039) (LPS) (median and IQR,  $P < 0.0001$ ). Similarly, LTA increased expression from 1106 MFI (959 – 1156) (Control) to 1889 MFI (1740 – 2012) (LTA 30 µg/ml) (median and IQR,  $P < 0.01$ ). Incubation with TNF for 6 h also significantly increased ICAM-1 expression (Supplementary Fig 1A).

In line with previous studies the TNF and LPS response was time-dependent (Supplementary Fig 2), and concentration dependent (data not shown).<sup>14</sup> Furthermore, the LPS response was dependent on *de novo* translation, as evidenced by the ability of cycloheximide (CHX) to block the uplift in ICAM-1 expression (Supplementary Fig 3). While ICAM-1 expression was shown to be maximal at 20 h (Supplementary Fig 2), our study focussed on ICAM-1 expression at 6 h to minimise the impact of neutrophil apoptosis at later time-points. To investigate ICAM-1 expression in apoptotic and non-apoptotic neutrophils, time-course experiments were performed in the presence and absence of LPS, TNF and LTA (Supplementary Fig 4). In agreement with previously published data,<sup>27–29</sup> we observed that all three mediators decreased neutrophil apoptosis significantly. Furthermore, neutrophil apoptosis was accompanied by loss of ICAM-1 expression; a finding that aligns with the molecular profile of a number of immunoglobulin superfamily members including PECAM-1, ICAM-3 and CD66b.<sup>30</sup>

To confirm the expression of ICAM-1 in unmanipulated neutrophils we used a ‘whole blood’ flow cytometry technique, which gates neutrophils based on their FSC/SSC and CD16 positivity.<sup>26</sup> Using this approach, ICAM-1 expression increased following incubation with LPS; control 795 (723 – 1084) to 1244 (1134 – 1708) (median and IQR,  $P < 0.05$ ; Fig 1C). Finally, using tandem mass spectrometry proteomics,<sup>31</sup> we detected ICAM-1 on neutrophils, with up-regulation following incubation with GM-CSF (Supplementary Fig 1B). This approach also detected ICAM-3 and ICAM-4 on the surface of neutrophils. ICAM-3 has been detected in human neutrophils, targeting them for efferocytosis by macrophages.<sup>32</sup> Of note, ICAM-4 has not been detected on neutrophils previously; its presence previously restricted to erythrocytes and macrophages.<sup>33</sup>

Consistent with these findings, an RNA transcriptomic study demonstrated ICAM-1 expression in neutrophils<sup>34</sup> (Supplementary Table 1), with 1.5-fold up regulation following incubation with GM-CSF for 6 h ( $P < 0.05$ ). This dataset also revealed basal expression of ICAM-2, -3 and -4 expression in neutrophils. To our knowledge ICAM-2 has so far only been detected on murine bone marrow neutrophils.<sup>35</sup>

Collectively, these data are in line with previous studies showing that ICAM-1 is present on human neutrophils and can be induced by inflammatory mediators.<sup>14,15</sup> Moreover, our findings now add LTA to the repertoire of inducers of neutrophil ICAM-1 expression. LTA has been shown to shed CD62L, stimulate IL-8 secretion, and inhibit neutrophil apoptosis.<sup>36</sup> As a component of gram-positive bacteria, it is tempting to speculate that LTA is responsible for the uplift in ICAM-1 following stimulation with *S. aureus*,<sup>19</sup> and thus have a modulatory role during pleural infections.

### **ICAM-1 expression on extravasated neutrophils**

Having found that inflammatory mediators can directly modulate ICAM expression, we next considered the expression of ICAM-1 on extravasated neutrophils. To investigate whether *in vivo* transmigration alters neutrophil ICAM-1 expression, neutrophils were isolated from two extravascular populations; the oral cavity of healthy volunteers and the pleural space of patients with a parapneumonic effusion or empyema. As shown in Fig 2A, ICAM-1 expression of neutrophils in the oral cavity was comparable to blood-derived neutrophils from healthy volunteers. However, neutrophils obtained from the pleural cavity (Supplementary Table 2), exhibit marked up-regulation of ICAM-1 expression compared to blood-derived neutrophils from healthy controls (3342 MFI (3036 – 5979) to 1092 MFI (844

– 1329) (median and IQR,  $P < 0.01$ ). Moreover, we observed this significant uplift in ICAM-1 expression comparing blood-derived neutrophils from four patients with a parapneumonic effusion compared to their counterparts obtained from the pleural cavity, suggesting that the neutrophils from these patients exhibit no systemic change in their levels of ICAM-1 (1411 MFI (1314 – 1561) to 3276 MFI (2872 – 4528) (median and IQR,  $P < 0.05$ ; Fig 2B). Of note, the pleural fluid-derived neutrophils displayed notable cytoplasmic vacuolation and few apoptotic cells as assessed by morphology (2.5% (0.6-4.2); median and IQR;  $n = 4$ ), shown in Fig 2C.

With the knowledge that pleural-fluid derived neutrophils displayed high levels of ICAM-1 expression, we next investigated the role of *in vitro* transmigration on ICAM-1 expression. To assess this, ICAM-1 expression in blood-derived neutrophils was assessed following transmigration across endothelial cell (EC)-coated inserts (Fig 2D). Neutrophil migration across EC-coated inserts increased ICAM-1 expression (1732 (1328 – 1943) compared to those incubated with IL-8 alone (824 (619-999) (median and IQR,  $P < 0.05$ ; Fig 2D).

Given that neutrophil transmigration did not fully recapitulate the extent of ICAM-1 uplift observed in pleural-derived neutrophils, we considered the contribution of inflammatory mediators in the pleural fluid. To assess this, cell-free pleural fluid samples were incubated with healthy volunteer blood derived neutrophils for 6 h and ICAM-1 expression was measured. Of the five cases, only donor D exhibited a discernible uplift in ICAM-1 expression (Fig 2E). To identify potential cytokines that mediate this uplift, we measured the expression of 10 pro-inflammatory mediators present in cell-free pleural fluid from each patient. As shown in Fig 2F, IL-1 $\beta$ , IL-2, IL-8, IL-10, IL-12, IL-13, and TNF levels were elevated in the pleural fluid obtained from donor D, compared to other donors. Notably,

donor D was a patient diagnosed with empyema, showing positive cultures for *S. aureus*. We excluded involvement of IL-1 $\beta$ , IL-6 and IL-8, as these cytokines did not alter ICAM-1 expression (Supplementary Fig 5). However, TNF is a likely candidate, given that the concentration in the pleural fluid (2.3 ng/ml) is sufficient to induce ICAM-1 expression (Supplementary Fig 1).

We cannot exclude the possibility of a combinatorial effect *in vivo* from endothelial transmigration, multiple cytokines and bacterial PAMPs present in the pleural fluid. Given that neutrophil ICAM-1 expression in the oral cavity was similar to blood-derived neutrophils this would suggest that transmigration itself may be insufficient to induce ICAM-1 expression *in vivo* and the presence of cytokines and/or bacterial components may be necessary to drive the increase in ICAM-1 expression. Alternatively, it is also possible that there are distinct processes involved in leaving the vasculature during homeostatic immune surveillance, as seen in the oral cavity, versus pathologic infection, in the case of pleural disease. This aligns with previous work demonstrating that there is a predominance of neutrophils with a low activation phenotype in the oral cavity of healthy individuals.<sup>37</sup>

### **ICAM-1<sup>high</sup> neutrophils exhibit enhanced phagocytic capacity**

ICAM-1 signalling is known to underlie phagocytosis in murine neutrophils.<sup>15</sup> To investigate whether ICAM-1 plays a role in human neutrophil phagocytosis, a high throughput phagocytosis assay was utilised, as previously described.<sup>26</sup> Given the established and consistent effect of LPS on ICAM-1-mediated phagocytosis in murine neutrophils,<sup>15</sup> we focussed on LPS as the stimulant rather than LTA.

LPS stimulation for 1 h significantly increased phagocytosis of *S. aureus* from  $1.64 \times 10^4$

( $1.39 \times 10^4 - 4.29 \times 10^4$ ) to  $4.84 \times 10^4$  ( $2.59 \times 10^4 - 7.09 \times 10^4$ ) (median and IQR,  $P < 0.05$ ; Fig 3A). LPS-stimulated neutrophils with lower ICAM-1 expression (25<sup>th</sup> centile) demonstrated reduced phagocytosis ( $3.73 \times 10^4$  ( $1.33 \times 10^4 - 3.98 \times 10^4$ )) relative to those with higher (75<sup>th</sup> centile) ICAM-1 expression ( $7.38 \times 10^4$  ( $4.33 \times 10^4 - 10.74 \times 10^4$ )) (median and IQR,  $P < 0.01$ ; Fig 3B). Similarly, in unstimulated cells, neutrophils with lower ICAM-1 expression demonstrate lower phagocytosis ( $1.20 \times 10^4$  ( $5.48 \times 10^3 - 2.73 \times 10^4$ )) relative to those with higher (75<sup>th</sup> centile) ICAM-1 expression ( $5.16 \times 10^4$  ( $2.33 \times 10^4 - 7.67 \times 10^4$ )) (median and IQR,  $P < 0.05$ ; Fig 3C).

Consistent with studies using murine neutrophils,<sup>15</sup> we found no difference in phagocytic uptake between the anti-ICAM-1 mAb and the isotype control (data not shown), indicating that extracellular integrin binding domain of ICAM-1 does not play a role in neutrophil phagocytosis. To determine if LPS-mediated enhancement of phagocytosis was dependent on ICAM-1 intracellular signalling, whole blood was incubated with a cell permeable peptide penetratin-ICAM-1 to block the downstream activation of ICAM-1 (Fig 3D). Despite the penetratin-ICAM-1 peptide reducing LPS induced phagocytosis from 1.89 (1.65 – 2.61) to 1.32 (1.01-1.86) (median and IQR), the decrease was not statistically significant. Of note, neither the penetratin-ICAM-1 peptide nor the scrambled peptide altered the up-regulation of ICAM-1 by LPS (Fig 3E).

In addition to phagocytosis, ICAM-1 signalling has been shown to facilitate reactive oxygen species (ROS) responses.<sup>15,38</sup> To investigate whether ROS generation was enhanced in ICAM-1<sup>high</sup> neutrophils, a dihydrorhodamine (DHR) probe was utilised in a whole blood assay (Fig 3F). In contrast with murine neutrophils, ICAM-1<sup>high</sup> human neutrophils did not exhibit significantly higher ROS production in either unstimulated or LPS stimulated

neutrophils.

Taken together, these data suggest that ICAM-1 intracellular signaling contributes to LPS-induced phagocytosis of *S. aureus*, but the involvement of additional receptors and signaling pathways remains to be determined. For example, LPS can act on neutrophil TLR4 receptors,<sup>39</sup> and downstream pathways of TLR4 receptors such as MAPK and PI-3Ks are also implicated in phagocytosis.<sup>40,41</sup>

## CONCLUDING REMARKS

In summary, our study identifies LTA as a novel modulator of ICAM-1 expression *in vitro* and demonstrates evidence of ICAM-3 and -4 expression in human neutrophils. Our results show that *in vivo* endothelial transmigration can induce ICAM-1 expression, whilst suggesting other factors (e.g. cytokines and/or PAMPs) also contribute to the ICAM-1<sup>high</sup> phenotype in inflammatory compartments. Moreover, this study also highlights distinctions between murine and human neutrophils, in terms of their differing reliance on ICAM-1 signaling for phagocytosis and ROS generation. Further studies are needed to unravel the pathways mediating ICAM-1 signalling in neutrophils from inflammatory sites before consideration of neutrophil ICAM-1 as a therapeutic target.

## FIGURE LEGENDS

**Figure 1. ICAM-1 expression in human neutrophils** (A) Neutrophils were incubated with or without LPS (10 ng/ml) for 6 h at 37°C and ICAM-1 expression measured by flow cytometry. Median and interquartile range from 15 independent experiments, \*\*\*\* $P < 0.0001$

by Mann-Whitney U test. Representative histogram of ICAM-1 expression in neutrophils after 6 h, showing isotype (grey), control (blue) and LPS (red). (B) Neutrophils were incubated with or without LTA (0.1 - 30 µg/ml) for 6 h at 37°C. Median and interquartile range from 5 independent experiments,  $**P < 0.01$  by Friedman and Dunn's posthoc test, compared to control. Representative histogram of ICAM-1 expression in neutrophils after 6 h, showing isotype (grey), control (blue) and LTA (red). (C) ICAM-1 expression following LPS (100 ng/ml) stimulation for 1 h, expression assessed using a whole blood flow cytometry assay. Median and interquartile range from 6 independent experiments,  $*P < 0.05$  by Mann-Whitney U test. Representative histogram of ICAM-1 expression in neutrophils after 6 h, showing isotype (grey), control (blue) and LPS (red).

**Figure 2. ICAM-1 expression in extravasated neutrophils** (A) Expression of ICAM-1 from peripheral blood-, oral cavity- and pleural cavity-derived neutrophils. Data show blood-derived and oral cavity-derived neutrophils from healthy volunteers (HV), and pleural fluid-derived neutrophils from patients with pleural effusions. ICAM-1 expression was measured by flow cytometry. Median and interquartile range,  $**P < 0.01$  by Kruskal-Wallis and Dunn's posthoc test. (B) Expression of ICAM-1 from peripheral blood-derived neutrophils and pleural cavity-derived neutrophils from four patients with a parapneumonic effusion (PE); patient ID F, G, H and I in Supplementary Table 2. ICAM-1 expression was measured by flow cytometry. Median and interquartile range from 4 independent experiments,  $*P < 0.05$  by Mann-Whitney U test. (C) Photomicrographs of neutrophils obtained from the pleural cavity (40 × and 100 × (inset) magnification. (D) Expression of ICAM-1 on blood-derived neutrophils following transmigration across HUVEC-coated transwell inserts. Migration across inserts was stimulated using IL-8 (10 ng/mL) in the lower chamber with an incubation period of 2 hours. Median and interquartile range from 4 independent

experiments,  $*P < 0.05$  by Mann-Whitney U test. (E) Blood-derived neutrophils were resuspended in RPMI1640 with cell-free pleural fluid from patients with parapneumonic effusions or empyema (donors A-E), or LPS alone (10 ng/ml) for 6 h. ICAM-1 expression was determined by flow cytometry. Median and interquartile range from 2 independent experiments. (F) Cytokine levels in cell-free pleural fluid, measured using a 10-plex cytokine profiling kit.

**Figure 3. Quantification of neutrophil phagocytosis and ROS production from ICAM-1<sup>high</sup> and ICAM-1<sup>low</sup> neutrophils in whole blood.** (A) Whole blood phagocytosis of neutrophils was assessed in the presence of LPS (100 ng/ml) or vehicle control following a 1 h incubation. Median and interquartile range from 9 independent experiments,  $*P < 0.05$  by Mann-Whitney U test. (B) Comparison of phagocytic index in the ICAM-1<sup>high</sup> populations (25th percentile) and ICAM-1<sup>low</sup> populations (75th percentile) from the LPS stimulated cells. Median and interquartile range from 9 independent experiments,  $**P < 0.01$  by Mann-Whitney U test. The flow cytometry gating strategy to identify ICAM-1<sup>high</sup> and ICAM-1<sup>low</sup> neutrophils is shown in Supplementary Fig 6. (C) Comparison of phagocytic index in the ICAM-1<sup>high</sup> populations (25th percentile) and ICAM-1<sup>low</sup> populations (75th percentile) from the unstimulated cells (CTL). Median and interquartile range from 9 independent experiments,  $*P < 0.05$  by Mann-Whitney U test. (D) Whole blood stimulated with LPS (100 ng/ml) for 1 h was incubated with a blocking penetratin peptide containing the C-terminal 16 aa of human ICAM-1 (BP) or a scrambled penetratin peptide (SP) for 1 h. Median and interquartile range from 8 independent experiments,  $***P < 0.001$  by Kruskal-Wallis and Dunn's posthoc test. (E) ICAM-1 expression measured by flow cytometry. Median and interquartile range from 6-8 independent experiments,  $*P < 0.05$  and  $**P < 0.01$  by Kruskal-Wallis and Dunn's posthoc test. (F) Whole blood was stimulated and ROS production of

neutrophils was assessed in the presence of LPS (100 ng/ml) or vehicle control for 1 h followed by the addition of DHR with or without pHrodo<sup>TM</sup> red *S. aureus* BioParticles, as described in Supplemental Methods. The flow cytometry gating strategy to identify ICAM-1<sup>high</sup> and ICAM-1<sup>low</sup> neutrophils is shown in Supplementary Fig 6. Median and interquartile range from 6 independent experiments, \* $P < 0.05$  and \*\* $P < 0.01$  by Kruskal-Wallis and Dunn's posthoc test.

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## Authorship

Contribution: M.V., N.F., A.J.T.W., E.N. and J.S performed experiments, analyzed data, and wrote the manuscript, with contributions from the other authors as appropriate; J.H., C.S., E.R.C., S.N. All authors discussed the results and commented on the manuscript.

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## Supplementary File

### Regulation of ICAM-1 in human neutrophils

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## **Supplementary Methods**

### **Proteomic plasma membrane profiling using tandem mass tags (TMT)**

Plasma membrane profiling was performed, as described previously.<sup>1</sup> Briefly,  $20 \times 10^6$  neutrophils were incubated with GM-CSF (10 ng/ml) for 0.5, 2 and 4 h or vehicle control (0 h). Cells were pelleted (260g, 5 min, 4°C) and resuspended in 1 ml of aminoxy-biotin (Insight Biotechnology) solution (50 pg/ml) reconstituted in 1 mM sodium periodate (Invitrogen-ThermoFisher, Carlsbad, CA, USA) with PBS (pH 6.7) and 1  $\mu$ l aniline (Sigma) per  $10 \times 10^6$  cells.

After 30 min rotation end over end at 4°C (protected from light), the reaction was quenched with 100  $\mu$ l of 10 mM glycerol per  $10 \times 10^6$  cells. Cells were washed three times with PBS and cell pellets stored at -80°C until protein isolation and peptide labeling. Protein was digested with Trypsin (Invitrogen-ThermoFisher) or Trypsin/LysC mix (Promega). One hundred percent of each peptide was labeled with TMT reagent (Invitrogen-ThermoFisher), and six fractions were generated from combined peptide samples by tip-based strong cation exchange.

Mass spectrometry was performed using an Orbitrap Elite or an Orbitrap Fusion and quantified tandem mass tag reporter ions from the MS3 scan. Peptides were identified and quantified using a Sequest-based in-house software pipeline. Peptide-spectral matches were filtered to a 1% false discovery rate (FDR) using linear discriminant analysis in conjunction

with the target-decoy method. The resulting data set was further collapsed to a final protein-level FDR of 1%. Protein assembly was guided by principles of parsimony.

### **Assessment of neutrophil apoptosis**

Neutrophils ( $5 \times 10^6$ /ml) were resuspended in IMDM supplemented with 10% autologous serum and incubated with LPS (10 ng/ml), TNF (10 ng/ml), LTA (30  $\mu$ g/ml) or vehicle control for 30 h at 37°C and 5% CO<sub>2</sub>. At each time-point, cells were harvested and apoptosis determined by flow cytometry using the Dead Cell Apoptosis kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) (Invitrogen-Thermofisher), according to manufacturer's instructions. To compare ICAM-1 expression between the apoptotic and non-apoptotic populations, cells were gated as non-apoptotic (annexin V<sup>-</sup>/PI<sup>-</sup>) or apoptotic (annexin V<sup>+</sup>/PI<sup>-</sup> and annexin V<sup>+</sup>/PI<sup>+</sup>).

### **Assessment of whole blood neutrophil ROS production**

Peripheral venous blood, anticoagulated in argatroban (150  $\mu$ g/ml, R&D Systems), was stimulated with LPS (100 ng/ml) or vehicle control for 1 h. All conditions were kept on a thermomixer at 300 rpm and 37°C before incubation with 20  $\mu$ g/ml pHrodo™ red *S. aureus* BioParticles (Invitrogen-Thermofisher) and Dihydrorhodamine-123 (DHR; Calbiochem) for 30 min. Cells were stained with anti-ICAM-1 BV421 (Clone HA58, BD Biosciences) and anti-CD16-Pacific blue and analyzed using an Attune NxT Acoustic Focusing Cytometer (Invitrogen-Thermofisher). ROS production was determined using FlowJo™ and calculated as the median fluorescence intensity, as described.<sup>2</sup>

## **Supplementary Figure Legends**

### **Supplementary Figure 1**

#### **Effect of TNF and GM-CSF on ICAM-1, -3, and -4 expression.**

(A) Neutrophils ( $5 \times 10^6$ /ml) were incubated with TNF (0.03-10 ng/ml) for 6 h at 37°C.

ICAM-1 expression was measured by flow cytometry. Median and interquartile range from 5 independent experiments, \* $P < 0.05$  by Friedman and Dunn's posthoc test, compared to

control. (B) Freshly isolated neutrophils were incubated with GM-CSF (10 ng/ml) at 37°C and plasma membrane profiling using tandem mass tags performed, as described in Methods.

Data show the relative intensity of ICAM-1, -3, and -4 expression on GM-CSF treated neutrophils at 0.5, 2 and 4 h, compared to control at 0 h. Data show the mean of a single experiment, representative of 2 experiments.

### **Supplementary Fig 2**

#### **Time course of ICAM-1 expression in neutrophils**

Neutrophils ( $5 \times 10^6$ /ml) were incubated with LPS (10 ng/ml), TNF (10 ng/ml), LTA (30  $\mu$ g/ml) or vehicle control for 30 h at 37°C. ICAM-1 expression was measured by flow cytometry. Data show the mean and SEM of 5 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$  by two-way ANOVA and Bonferroni's posthoc test, compared to control.

### **Supplementary Figure 3**

#### **Effect of cycloheximide on LPS-induced ICAM-1 expression.**

Neutrophils ( $5 \times 10^6$ /ml) were treated with LPS (10 ng/ml) with or without the translational inhibitor cycloheximide (CHX) (1  $\mu$ g/ml) for 6 h. Median and interquartile range from 5 independent experiments,  $*P < 0.05$  by Mann-Whitney test, compared to LPS alone.

#### **Supplementary Figure 4**

##### **ICAM-1 expression in apoptotic and non-apoptotic neutrophils**

Neutrophils ( $5 \times 10^6$ /ml) were incubated with LPS (10 ng/ml), TNF (10 ng/ml), LTA (30  $\mu$ g/ml) or vehicle control for 30 h at 37°C. ICAM-1 expression and neutrophil apoptosis was assessed by flow cytometry using an Annexin-V/PI assay. (A) Time-course of neutrophil apoptosis. (B-E) ICAM-1 expression in non-apoptotic and apoptotic neutrophils stimulated by PBS control, control (B), LPS (C), LTA (D), or TNF (E). Data show the mean and SEM of 5 independent experiments.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  and  $****P < 0.0001$  by two-way ANOVA and Bonferroni's posthoc test, compared to control.

#### **Supplementary Figure 5**

##### **Effect of IL-1 $\beta$ , IL-6 and IL-8 on ICAM-1 expression in human neutrophils. (A)**

Neutrophils ( $5 \times 10^6$ /ml) were incubated with LPS (10 ng/ml), or IL-1 $\beta$  (100 ng/ml) for 6 h at 37°C. ICAM-1 expression was measured by flow cytometry. Data show the median and interquartile range of 4 experiments (LPS) and 4 experiments (IL-1 $\beta$ ),  $*P < 0.05$  by Mann-Whitney U test. (B) Neutrophils were incubated with LPS (10 ng/ml) or IL-6 (1-100 ng/ml), for 6 h at 37°C. ICAM-1 expression was measured by flow cytometry. Data show the median and interquartile range of 3 independent experiments. (C) Neutrophils were incubated with LPS (10 ng/ml) or IL-8 (1-100 ng/ml), for 6 h at 37°C. ICAM-1 expression was measured by flow cytometry. Data show the median and interquartile range of 2 experiments.

## Supplementary Figure 6

### Flow cytometry gating strategy to identify ICAM-1<sup>high</sup> and ICAM-1<sup>low</sup> neutrophils.

Whole blood was stimulated with or without LPS (10 ng/ml) as described in the Materials and Methods. Plots show representative histograms for control (A) and LPS (B) stimulated whole blood, with backgating shown in the adjacent side panels. Neutrophils were identified on CD16 positivity (upper right-hand panels) and FSC/SSC profile (lower right-hand panels). The neutrophil population was plotted as a histogram to view ICAM-1-BV421 intensity. Cross-hairs were placed to set the thresholds for the ICAM-1<sup>high</sup> (upper 25% centile) and ICAM-1<sup>low</sup> (lower 75% centile) neutrophils. These gates were applied to all samples.

## Supplementary Table 1

### RNA transcriptomic analysis comparing ICAM-1, -2, -3, and -4 expression in control and GM-CSF treated human neutrophils.

Neutrophils were incubated with GM-CSF (10 ng/ml) or vehicle control for 6 h, before RNA transcriptomic analysis, as described.<sup>3</sup> Data from the National Center for Biotechnology Information Gene Expression Omnibus (GEO), GEO series accession number GSE76293 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76293>). Data show 10 experiments (Control) and 10 experiments (GM-CSF). Analyzed using GEO2R,<sup>4</sup> with logFC in control samples compared to GM-CSF samples.

Probe ID	Gene symbol	logFC	adj.P.Val
215485_s_at	ICAM-1	0.31575868	<b>0.0288</b>
202637_s_at	ICAM-1	0.61048746	0.0699
213620_s_at	ICAM-2	-0.30922612	0.7140
204949_at	ICAM-3	-0.06912458	0.7400

207194\_s\_at ICAM-4 -0.04367308 0.9640

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## Supplementary Table 2

### Diagnosis of patients.

Pleural fluid-derived neutrophils were obtained from patients and their diagnosis recorded.

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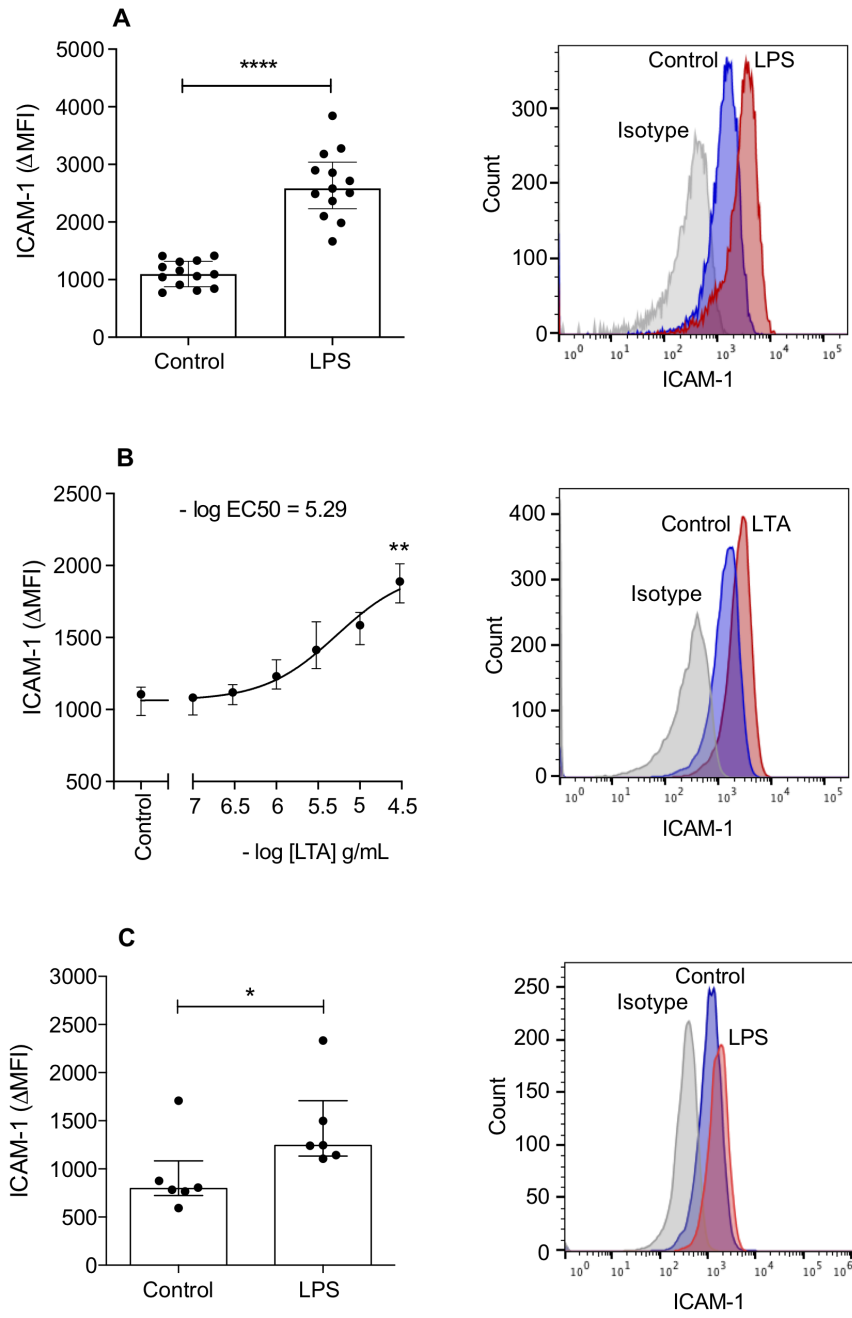
Patient ID	Diagnosis
A	Squamous cell carcinoma
B	Loculated (L) pleural effusion: likely empyema
C	Empyema
D	Empyema
E	Empyema (Sarcoidosis and chronic (L) effusion, recent flu B)
F	Empyema
G	Complex septated para-pneumonic effusion
H	Empyema
I	Empyema

## REFERENCES

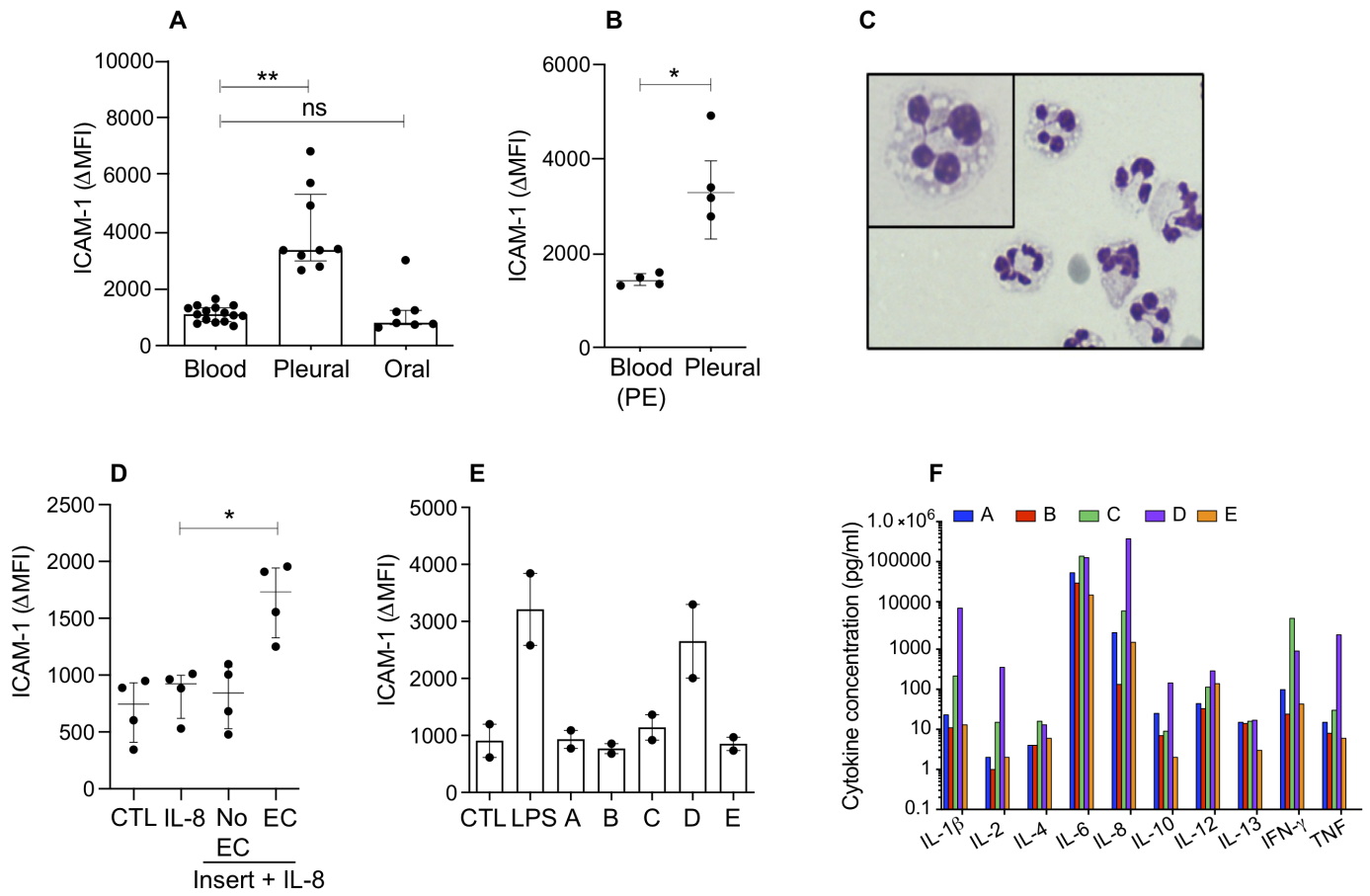
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Figure 1



**Figure 2**



**Figure 3**

