A simple skin blister technique for the study of *in vivo* transmigration of human leukocytes

Lisa Davidsson[a], Lena Björkman[a], Karin Christenson[a], Mikael Alsterholm[b], Charlotta Movitz[c], Fredrik B. Thorén[c], Anna Karlsson[a], Amanda Welin[a], Johan Bylund[a]*.

[a]The Phagocyte Research Group, Department of Rheumatology and Inflammation Research, and [b]Department of Dermatology and Venereology, and [c]Sahlgrenska Cancer Center, the Sahlgrenska Academy at the University of Gothenburg, Sweden.

Running title: A human *in vivo* model of aseptic inflammation

Key words: inflammation, transmigration, neutrophil, exudate, cytokines

*Corresponding author

Postal address: Box 480, 405 30 Gothenburg, Sweden

Telephone: +463124685

E-mail address: johan.bylund@rheuma.gu.se

Abstract

The study of human leukocytes is almost exclusively conducted using cells isolated from peripheral blood. This is especially true for neutrophils, despite the fact that these cells are of main (pathological) importance in extravascular tissues upon e.g., infection and/or tissue damage. The journey from circulation to tissue is typically associated with a number of cellular changes, making the cells primed, or hyper-responsive, and in many aspects distinct from the cells present in circulation. Models to obtain *in vivo* transmigrated leukocytes from human tissue are available, but not widely used. We here describe an easy-to-use model for the study of local inflammation, stemming from limited tissue damage, which can be used to isolate viable and functional leukocytes. The model is based on the generation of aseptic skin blisters, formed by the application of negative pressure, and allows for investigations of the cellular infiltrate as well as of soluble mediators present in the exudate. We believe that this method, combined with modern analysis equipment suitable for small volumes and cell numbers, could be of great use for increasing our understanding of the nature and function of leukocytes that have left circulation and transmigrated to inflamed tissues.

1. Introduction

The typical course of events during acute inflammation is initiated by the recognition of danger signals, e.g., molecules derived from microbes or damaged self, in the tissues. Such innate recognition triggers a variety of processes that pave the way for the infiltration of inflammatory cells that are to neutralize the perceived threat. The inflammatory cells recruited from the blood to sites of acute inflammation are typically neutrophils, followed by monocytes and certain lymphocytes. In humans, the most common source of cells for *in vitro* studies of inflammatory processes is peripheral blood. However, the journey from circulation to tissue is associated with a number of cellular changes, most notably represented by alterations in the composition and/or abundance of cell surface receptors (Paccaud et al., 1990; Karlsson et al., 1998; Seely et al., 2002). Hence, tissue neutrophils are typically primed, or hyper-responsive, and in many aspects distinct from the cells in circulation (Zarember and Kuhns, 2011). Despite this, surprisingly little is known about the nature and function of tissue neutrophils that have undergone *in vivo* transmigration.

The available methods for obtaining *in vivo* transmigrated neutrophils, or other leukocytes, have different advantages and disadvantages. Evaluation of data on neutrophils from “natural” sites, e.g., pus (Kuhns et al., 1995), saliva (Lukac et al., 2003), urine (our unpublished observation), or BAL fluid (Hustinx et al., 1998), is complicated by the presence of microbes (the infection that initiated the inflammatory response, and/or an abundant normal microbial flora) and the dependence on availability of patient material. The latter is also true for, e.g., synovial fluid from rheumatic patients, where an underlying chronic inflammation is likely to complicate interpretation of data. Furthermore, most natural sources of tissue neutrophils suffer from the

drawback that it is often impossible to determine when the acute inflammation was initiated; information that may be crucial for proper study of e.g., short-lived neutrophils.

More controlled means to obtain tissue neutrophils from an aseptic source often involve the skin and the method underlying the bulk of our knowledge on *in vivo* transmigrated neutrophils is the skin chamber model (Follin and Dahlgren, 2007). This approach is based on the creation of skin blisters, caused by the application of relatively low negative pressure that causes the epidermis of the skin to detach from the dermis (Kiistala and Mustakallio, 1964) and expose dermal papillae on the blister floor, without concomitant bleeding. The blister roofs are subsequently removed and replaced by collection chambers made from acrylic plastic that are filled with autologous serum or an alternative source of chemoattractants (Follin and Dahlgren, 2007). The serum-filled chambers are then left on the subject and after 18-24 hours the serum is filled with transmigrated cells.

In this report we describe and characterize a simplified and useful version of the well-known skin chamber model that can be used to obtain and study *in vivo* transmigrated human leukocytes in parallel to the soluble inflammatory mediators present in the exudate. We have utilized the vacuum-derived skin blisters mentioned above, but without removing the skin roof and applying collection chambers. Instead, the cells are allowed to migrate into the aseptic, undisturbed, and natural blister exudate, from which they can be collected directly using a micropipette. In comparison to the skin chamber technique, our blister technique has the benefit of creating an inflammatory milieu that is less artificial than the plastic skin chambers filled with activated serum. Despite the relatively low exudate volumes and cell numbers obtained, a wealth of

knowledge on the nature of leukocytes after *in vivo* transmigration can still be generated, especially when used in combination with micro-assays developed for small samples. We believe that the technique described here is simple and convenient enough to suit most labs with an interest in leukocyte biology and hope that it can increase the available knowledge of the behaviour of these cells after *in vivo* transmigration.

2. Materials and Methods

2.1 Study subjects

Exudated human leukocytes as well as leukocytes from peripheral blood were obtained from healthy volunteers after obtaining informed consent. The study was approved by the Regional Ethical Board (Sweden; No. 543-07).

2.2 Creation of skin blisters

To generate skin blisters we used acrylic custom-made suction chambers, 40 mm in overall diameter and 18 mm high. The bottom surfaces of the chambers were flat and had three holes with a diameter of 5 mm, situated 8 mm apart in the shape of a triangle. Similar suction chambers are commercially available from e.g., http://www.electdiv.com. The suction chambers were connected to a portable handheld vacuum pump (Mityvac II, Mityvac, St. Louis, MO) with tubing (Fig. 1) and fixed to the broadest volar part of the forearm with surgical tape after which negative pressure was applied (approximately 300-400 mmHg). We used two chambers, coupled in parallel, resulting in a total of 6 blisters per subject. Once negative pressure was applied with a satisfactory seal, the chambers were covered with an elastic bandage. With a reasonably good seal, additional pumping to sustain a negative pressure of 300-400 mmHg was required only occasionally. In order to promote superficial circulation, the subjects were asked to wear a sweater to keep the arm warm during the procedure.

Negative pressure caused the epidermis to separate from the basal membrane resulting in the formation of superficial blisters (Fig. 1) within 1.45h, after which the suction chambers were removed (this time point is referred to as T0). At this point, all blisters were visually inspected to

ensure that exudates were free from signs of bleeding. Minor bleeding occasionally occurred in isolated blisters, most likely due to excessive negative pressure, and such blisters were not used for further analyses. After formation, the blisters were protected with small plastic covers (Fig. 1) kept in place by careful wrapping in a latex-free self-adherent wrap (Coban LF, 3M). This procedure kept the blisters intact and allowed the subject to resume normal activity without risking blister damage before fluid collection.

To empty the blister content we used a sterile needle (23G1" 0.6x25 mm; BD Microlance) to puncture the blister roof after which the fluid was aspirated using a 20 µl micropipette. To collect maximal volume of blister fluid, light pressure was applied to the blister roof, pushing the fluid towards the pipette tip. Collected blister fluid was weighed and kept on ice in a special type of tube (Protein LoBind tubes, 1.5 ml; Eppendorf, Cat no. 022431081) to prevent cells from adhering to the tube walls. When developing the method, we first used standard Eppendorf tubes, resulting in low cell recovery after incubation of cells (even incubations on ice). The Protein LoBind tubes were thus used throughout this study for all incubations. An additional precaution used in order to minimize cell adherence to plastic was chelation of calcium ions, by including EDTA (100 µM final concentration) in buffers used for dilution and washing. Since calcium ions are crucial for proper leukocyte function (e.g., degranulation and phagocytosis), EDTA was never used for functional assays.

Blister leukocytes were not separated from the exudate fluid prior to analyses, and the term blister fluid refers to both leukocytes and fluid obtained from the blisters.

2.3 Separation of leukocytes from peripheral blood

For some experiments, as controls, we used peripheral blood leukocytes from the same volunteer that was subjected to the blister method. Blood was drawn into heparinized tubes, erythrocytes were removed by Dextran (Pharmacosmos, Holbaek, Denmark) sedimentation and hypotonic lysis, after which the leukocytes were washed twice in Krebs–Ringer phosphate buffer (KRG; pH 7.3) containing glucose (10 mM). The cells were stored in KRG with Ca^{2+} (1 mM) on ice until use.

2.4 Analysis of blister leukocytes and soluble mediators over time

Blister fluids from each subject were collected at different time points (0, 2, 4, 6, 8, and 24h) after removal of negative pressure and were diluted 1:20 in PBS with EDTA (100 µM final concentration). To determine the cellular composition in the blister fluid samples, samples were stained, without prior washing, with a mouse anti-human CD45-APC antibody (Abcam) on ice for 30 minutes to ensure good separation of neutrophils, monocytes and lymphocytes when analyzed by flow cytometry (Accuri C6, Becton Dickinson). Events negative for CD45 were considered non-leukocytes and excluded from further analyses. Relative abundance of neutrophils, monocytes, and lymphocytes in each sample was calculated as percentage of total number of CD45-positive events. To minimize the handling of cells, no washing was performed prior to analysis. We optimized the analysis so that as little as 3 µl of blister fluid was utilized and Fig. 2A display all events recorded during acquisition a sample volumes corresponding to 1 µl blister fluid.

The remaining blister fluid was frozen (-80°C) and later evaluated for cytokine content (IL-1β, IL-6, IL-8, IL-10, IL-12, IL-17, GM-CSF, TNF-α, IFN-γ, IP-10) with a Bio-plex pro cytokine assay 10-Plex group 1 (Bio-Rad Laboratories), according to the manufacturer´s description. All blister fluids were diluted to the same volume (55 µl) prior to analysis and the dilution factor for every sample was multiplied to the resulting values for each cytokine.

2.5 Expression of surface markers

Blister fluid was diluted 1:10 in PBS alongside peripheral blood leukocytes from the same donor (diluted to match the cell density in the blister fluid), and incubated either at 37° for 20 min in the presence of TNF-α (10 ng/ml; Sigma-Aldrich), or on ice. All samples were stained with fluorophore-conjugated antibodies; either mouse anti-human L-selectin (CD62L) or mouse anti-human CR3 (CD11b) (both from Becton Dickinson) on ice for 30 minutes. Samples were then directly analyzed by flow cytometry without washing.

2.6 Viability assay

Analyses of cell death were performed essentially as described (Christenson et al., 2012). Five µl of blister fluid was diluted 10 times in Annexin V buffer with Annexin V-FLUOS (Roche Diagnostics), incubated on ice for 15 min, before addition of the nucleic acid dye To-Pro-3 (Invitrogen) and immediately analyzed by flow cytometry.

2.7 Intracellular staining

Intracellular olfactomedin 4 (OLFM4) and neutrophil gelatinase-associated lipocalin (NGAL) were stained using indirect immunofluorescence. Isolated blood leukocytes or blister fluid were

fixed using 4 % paraformaldehyde and permeabilized using ice cold acetone and methanol at a 1:1 ratio for 5 min. Blocking was performed using 10 % normal goat serum (Sigma-Aldrich) and 2 % bovine serum albumin. Subsequently, samples were incubated with primary polyclonal rabbit anti-human OLFM4 antibody (Abcam, 5 µg/ml) and monoclonal mouse anti-human NGAL antibody (Abcam, 5 µg/ml). After washing, samples were incubated with secondary Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 647-conjugated goat anti-mouse IgG ($F(ab')_2$ fragment (Molecular Probes, both 5 µg/ml), washed again and analyzed using flow cytometry.

2.8 Phagocytosis assay

For phagocytosis assays, *Staphylococcus aureus* strain RN4220 harboring the pCN-GFP plasmid for constitutive expression of green fluorescent protein (GFP) (a kind gift from Maria Lerm, Linköping University) was used. The live bacteria were enumerated using a flow cytometer, and added to T6 blister fluid at a multiplicity of infection (MOI) of 5. Phagocytosis was allowed to proceed for 15 min at 37^{o}C, or on ice, before the cells were fixed in 3 % PFA, and analyzed using flow cytometry. Based on FSC and SSC, a gate was set that included both monocytes and neutrophils (=phagocytes). Cells displaying green fluorescence above background levels were classified as positive for association with bacteria. In order to visually evaluate whether the bacteria associated with the cells had been internalized or merely attached to the cell surface, an imaging flow cytometer (ImageStream^{X}, Amnis) was used. Before analysis, APC-conjugated anti-human CD45 antibody (as above) and DAPI (for DNA staining, 60 nM) were added to the samples. IDEAS software v. 5.0 was used for data analysis. Firstly, cells in focus were gated,

followed by identification of phagocytes based on CD45-positivity and size. Phagocytes that were associated with bacteria were selected based on GFP intensity above background levels.

3. Results

3.1 Different leukocyte subsets appear in the blister fluid over time

Blisters were formed on the forearm of healthy volunteers as described in Materials and Methods. After 1.45h of negative pressure the blister fluid was collected, incubated with anti-CD45-antibody and immediately analyzed for leukocyte content by flow cytometry. These fresh blisters showed an almost complete absence of CD45 positive cells (leukocytes; Fig. 2) indicating that no leukocytes had infiltrated the blisters at this time point (T0). The CD45 negative events in the blister fluid comprised a mixture of anuclear debris, cell fragments and occasional erythrocytes as determined by imaging flow cytometry (not shown). Worth noting is that such CD45 negative events were present already at T0 and their relative abundance did not change over time. Blister fluid collected at later time points, between 6 and 24 hours after removing the vacuum pump, typically contained leukocytes that based on their granularity and CD45 staining were identified as three distinct cell populations; neutrophils, monocytes and lymphocytes (Fig. 2A).

Based on the flow cytometry analysis, we performed a kinetic study of the infiltration of leukocytes into the skin blisters. Blister fluids from five subjects were collected at different time points (0, 2, 4, 6, 8, and 24h). The volume of blister fluid retrieved was similar over time (mean±SD 18.7 ±6.6 µl, n=27), but the total leukocyte counts (CD45 positive events) increased over the first 6-8 hours, with significant variability, after which the numbers were consistent up to T24 (Fig. 2B). The leukocyte counts in early blisters (T0-T4) were too low to enable further analysis, but in older blisters we determined the relative abundance of neutrophils, monocytes, and lymphocytes based on CD45 staining and granularity (as seen in Fig. 2A). True to the text

book view of acute inflammation, neutrophils dominated in the early phase, monocytes appeared later, and a distinct lymphocyte population was present only in T24 blisters (Fig. 2C).

We next monitored the viability of blister neutrophils by using a combination of Annexin V and a cell impermeable nucleic acid dye that only transverses membranes of necrotic cells (To-Pro-3). Viability of the transmigrated neutrophils was close to >95% at T6 and only occasional cells were classified as apoptotic (Annexin V positive / To-Pro-3 negative) or necrotic (Annexin V positive / To-Pro-3positive) by flow cytometry (Fig. 3). In T24 blisters, apoptosis was slightly increased whereas necrosis was negligible also at this time point (Fig. 3).

3.2 Levels of inflammatory mediators in blister fluid varies over time

We then analyzed the blister fluid for soluble inflammatory mediators at different time points, using multiplex analysis. For IL-1β and IL-6, levels increased rapidly, peaked at T8, and thereafter declined slowly (Fig. 4). The chemokine IL-8 showed a similar increase, peaking at T8 but remaining high until T24 (Fig. 4). Concentrations of TNF-α were highly elevated as compared to serum levels already at T0, increased to T2 and then declined to almost undetectable levels at T24 (Fig. 4). We also measured IL-10, IL-12, IL-17, GM-CSF, IFN-γ, and IP10, and the levels of these mediators either remained relatively constant over T0-T24 and/or were not markedly different from serum levels (Supplementary figure 1). In conclusion, these data show the power of using multiplex cytokine assays for small sample volumes, and gives a broad picture of the inflammatory milieu in the skin blisters.

3.3 Blister neutrophils exhibit a primed phenotype as shown by exposure of surface markers

The leukocyte's journey from circulation to tissue is typically associated with a number of cellular changes, most notably represented by alterations in the composition of cell surface receptors (Condliffe et al., 1998). To analyze the expression of surface molecules, blister fluid alongside peripheral blood leukocytes from the same donor were incubated at either at 37° for 20 min in the presence of the priming agent TNF-α, or on ice. All samples were stained with for CR3 or L-selectin and analyzed by flow cytometry.

Resting neutrophils obtained from peripheral blood are low in CR3 and high in L-selectin ($CR3^{low}$ $L\text{-}selectin^{high}$) exposure (Fig. 5). Priming *in vitro* by incubation with recombinant TNF-α resulted in a primed phenotype which is $CR3^{high}L\text{-}selectin^{low}$ (Fig. 5A). Blister neutrophils (T6, not shown; T24, Fig 5A) had a primed phenotype, $CR3^{high}$ $L\text{-}selectin^{low}$, indicating that these tissue neutrophils show a primed phenotype *in vivo*. Addition of TNF-α to blister cells did not further alter expression levels of L-selectin or CR3 (not shown) indicating that the cells have reached an end-stage phenotype with regard to priming.

Since the T24 blisters contained a distinct monocyte population, and the labelling for surface markers was done on the entire inflammatory infiltrate, we also analyzed these markers on monocytes. In analogy with neutrophils, the transmigrated monocytes were $CR3^{high}$ $L\text{-}selectin^{low}$ as compared to peripheral blood monocytes (Fig. 5B).

3.4 Analysis of intracellular markers – OLFM4 is stably present in blister neutrophils

We next stained for intracellular antigens in the blister leukocytes and found that permeabilization made gating of the neutrophil population using only size and granularity more

difficult. We therefore included labelling of the specific granule protein gelatinase-associated lipocalin (NGAL), an often used neutrophil-specific marker. We detected NGAL in the majority of events in the main population after permeabilization of T6 blister cells (Fig. 6A).

Another specific granule protein, olfactomedin 4 (OLFM4), was recently described to be expressed only in a subset (10-40 %) of circulating human neutrophils (Clemmensen et al., 2012) despite the fact that OLFM4 mRNA was found in 100% of immature myelocyte/metamyelocytes from bone marrow (Clemmensen et al., 2012). Not much is known about the function(s) of this protein in neutrophils and whether OLFM4 positive and OLFM4 negative neutrophils differ in functionality. We took advantage of the skin blister model to investigate if OLFM4 was expressed differently in blood and tissue neutrophils. As expected, the fraction of OLFM4 expressing neutrophils (NGAL positive) in blood varied between donors, but for each donor the relative abundance of OLFM4 positive neutrophils was not markedly different between blood and T6 blisters (Fig. 6B).

3.5 Functional analysis of blister neutrophils –the process of phagocytosis is intact

In order to investigate whether the transmigrated blister cells could be used in functional assays, phagocytosis experiments were carried out on these cells. Blister leukocytes were incubated with GFP-expressing *S. aureus* for 15 min at 37^{o}C, or on ice, before the cells were fixed and analyzed for association with bacteria using regular flow cytometry as well as imaging flow cytometry. The latter technique generates fluorescent microscopic images of cells in flow and was used here to distinguish between cells with extracellularly bound bacteria and cells having internalized the prey. The blister phagocytes were clearly functional as revealed by the flow cytometry based

quantification of GFP-positive cells (Fig. 7A). Imaging flow cytometry further confirmed that when incubation had taken place on ice bacteria were typically bound to cell surfaces, whereas the majority of bacteria were internalized when the experiment was carried out at 37°C (Fig. 7B). These data show that competent phagocytes are retrieved from the blisters and that it is possible to use these cells also in functional assays.

4. Discussion

In this report we describe and characterize a simple and useful technique to obtain and study *in vivo* transmigrated human leukocytes in parallel to the soluble inflammatory mediators present in the exudate. The technique described is based on the formation of skin blisters identical to those employed for the more widely used skin chamber methodology (Follin et al., 1991; Follin and Dahlgren, 2007) and in comparison to that technique the cellular yield is of course much lower. The cellular yield (and exudate volume) in the blisters is also significantly lower than what is reported with blisters created using the Spanish Fly venom, cantharidin (Day et al., 2001). However, despite the relatively low exudate volumes and cell numbers obtained using the presented blister technique, a wealth of knowledge on the nature of leukocytes after *in vivo* transmigration can still be generated, especially when used in combination with micro-assays developed for small samples. A significant advantage in this respect is the use of multiplex particle-based flow cytometric assays that enable the simultaneous measurements of multiple cytokines from very limited sample volumes (Vignali, 2000); we routinely analyzed 10 cytokines in as little as 4 µl blister fluid. Similarly, flow cytometric measurements can be performed on very limited sample volumes with small cell numbers. Our standard set-up used for following the arrival of different leukocyte subsets to the blisters was run using only 3 µl blister fluid, which generated clear data from T6 and older blisters. The analyses were run on a flow cytometer (Accuri C6) that operates with a low-pressure pumping system, without vacuum, allowing for the use of any kind of tubes. In addition, all analysis settings can be applied to samples after acquisition which is a major advantage when attempting to analyze a limited number of cells for as many parameters as possible. For more complex staining protocols, especially those involving

intracellular markers or the use of multiple antibodies, repeated washing steps invariably led to loss of cells and made it necessary to start with larger volumes of blister fluid.

One major advantage of the described skin blister technique is its simplicity, both for investigators and the study subjects. With regard to convenience for the volunteering subject bearing the blisters, this technique is much less bothersome as compared to the skin chamber method. The subjects are mobile during the time from blister formation to the time point chosen for blister emptying. Only during the actual blister formation, the subject experiences a slight immobilization while carrying the hand held vacuum pump, and in addition a minor discomfort, a slight itching, may be experienced. After the blisters have formed and are appropriately protected from external pressure/damage, the subjects are free to move around and recommence on regular activities without discomfort. The technique should thus be relatively easy to use on various patient groups where the study of aseptic inflammatory exudates is of interest.

The blister technique also has the benefit of creating an inflammatory milieu that is less artificial than e.g., plastic skin chambers filled with activated serum, and also avoids the use of exogenous irritants. Although the skin chamber method can be adapted so that, e.g., monocytes can be studied (Paulsson et al., 2009), the normal 24 hour protocol with autologous serum gives rise to a >90% pure neutrophil population (Follin and Dahlgren, 2007). In the skin blisters, neutrophils dominate during the early phase, but at time points where the cellular infiltrates were large enough for *in vitro* analysis, neutrophils always existed in the presence of a significant "contamination" of monocytes and lymphocytes. On the one hand, this is beneficial since it reflects the situation most often encountered in inflamed tissue. On the other hand, it makes

evaluation of bulk-assays more problematic. We therefore relied primarily on cellular assays where the read-out can be restricted to specific leukocyte subset using gating strategies. Although we have not used it here, we suspect that a variety of microscopic techniques and live cell imaging approaches could be well suited to study the character and function of blister leukocytes.

The cellular analyses described here are limited to regular flow cytometry and imaging flow cytometry, with which we show that it is possible to analyze cells with regards to extracellular markers, intracellular markers as well as functionality. Our data show that blister neutrophils and monocytes display an altered phenotype as compared to peripheral blood cells, $CR3^{high}$ L-$selectin^{low}$, typical for primed phagocytes and similar to that of phagocytes after *in vivo* transmigration to skin chambers (Sengelov et al., 1995; Karlsson et al., 1998; Christenson et al., 2011; Zarember and Kuhns, 2011). Also *in vitro* studies, using Transwell set-ups (with our without endothelial monolayers) that are far less complex than the *in vivo* situation, have reported similar signs of degranulation in transmigrated neutrophils (Hennigan et al., 1999; Paulsson et al., 2010). A novel finding of this paper is that OLFM4 is present to a similar extent in transmigrated and blood neutrophils. This protein was recently shown to be expressed only in a subset of neutrophils in circulation and our data show that the proportion OLFM4 positive neutrophils, which differs from donor to donor (Clemmensen et al., 2012), remains constant after *in vivo* transmigration to skin blisters.

The *in vitro* study of human neutrophils is almost always conducted on cells isolated from peripheral blood, despite the fact that these cells are of main importance after they have left

circulation and transmigrated to tissues. We believe that the technique described here is simple and convenient enough to suit most labs with an interest in leukocyte biology and hope that it can increase the available knowledge of the behaviour of these cells after *in vivo* transmigration.

References

Christenson, K., Bjorkman, L., Karlsson, J., Sundqvist, M., Movitz, C., Speert, D.P., Dahlgren, C. and Bylund, J., 2011, In vivo-transmigrated human neutrophils are resistant to antiapoptotic stimulation. Journal of leukocyte biology 90, 1055-63.

Christenson, K., Thoren, F.B. and Bylund, J., 2012, Analyzing cell death events in cultured leukocytes. Methods Mol Biol 844, 65-86.

Clemmensen, S.N., Bohr, C.T., Rorvig, S., Glenthoj, A., Mora-Jensen, H., Cramer, E.P., Jacobsen, L.C., Larsen, M.T., Cowland, J.B., Tanassi, J.T., Heegaard, N.H., Wren, J.D., Silahtaroglu, A.N. and Borregaard, N., 2012, Olfactomedin 4 defines a subset of human neutrophils. Journal of leukocyte biology 91, 495-500.

Condliffe, A.M., Kitchen, E. and Chilvers, E.R., 1998, Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin Sci (Lond) 94, 461-71.

Day, R.M., Harbord, M., Forbes, A. and Segal, A.W., 2001, Cantharidin blisters: a technique for investigating leukocyte trafficking and cytokine production at sites of inflammation in humans. Journal of immunological methods 257, 213-20.

Follin, P. and Dahlgren, C., 2007, A skin chamber technique as a human model for studies of aseptic inflammatory reactions. Methods Mol Biol 412, 333-46.

Follin, P., Wymann, M.P., Dewald, B., Ceska, M. and Dahlgren, C., 1991, Human neutrophil migration into skin chambers is associated with production of NAP-1/IL8 and C5a. European journal of haematology 47, 71-6.

Hennigan, S.M., Wang, J.H., Redmond, H.P. and Bouchier-Hayes, D., 1999, Neutrophil heat shock protein expression and activation correlate with increased apoptosis following transmigration through the endothelial barrier. Shock 12, 32-8.

Hustinx, W.N., Van Kessel, C.P., Heezius, E., Burgers, S., Lammers, J.W. and Hoepelman, I.M., 1998, Effects of granulocyte colony-stimulating factor (G-CSF) treatment on granulocyte function and receptor expression in patients with ventilator-dependent pneumonia. Clinical and experimental immunology 112, 334-40.

Karlsson, A., Follin, P., Leffler, H. and Dahlgren, C., 1998, Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils. Blood 91, 3430-8.

Kiistala, U. and Mustakallio, K.K., 1964, In-Vivo Separation of Epidermis by Production of Suction Blisters. Lancet 1, 1444-5.

Kuhns, D.B., Long Priel, D.A. and Gallin, J.I., 1995, Loss of L-selectin (CD62L) on human neutrophils following exudation in vivo. Cellular immunology 164, 306-10.

Lukac, J., Mravak-Stipetic, M., Knezevic, M., Vrcek, J., Sistig, S., Ledinsky, M. and Kusic, Z., 2003, Phagocytic functions of salivary neutrophils in oral mucous membrane diseases. Journal of oral pathology & medicine 32, 271-4.

Paccaud, J.P., Carpentier, J.L. and Schifferli, J.A., 1990, Exudation induces clustering of CR1 receptors at the surface of human polymorphonuclear leukocytes. Biochemical and biophysical research communications 172, 1203-8.

Paulsson, J.M., Held, C., Jacobson, S.H. and Lundahl, J., 2009, In vivo extravasated human monocytes have an altered expression of CD16, HLA-DR, CD86, CD36 and CX(3)CR1. Scandinavian journal of immunology 70, 368-76.

Paulsson, J.M., Jacobson, S.H. and Lundahl, J., 2010, Neutrophil activation during transmigration in vivo and in vitro A translational study using the skin chamber model. Journal of immunological methods 361, 82-8.

Seely, A.J., Naud, J.F., Campisi, G., Giannias, B., Liu, S., DiCarlo, A., Ferri, L.E., Pascual, J.L., Tchervenkov, J. and Christou, N.V., 2002, Alteration of chemoattractant receptor expression regulates human neutrophil chemotaxis in vivo. Annals of surgery 235, 550-9.

Sengelov, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C. and Borregaard, N., 1995, Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol 154, 4157-65.

Vignali, D.A., 2000, Multiplexed particle-based flow cytometric assays. Journal of immunological methods 243, 243-55.

Zarember, K.A. and Kuhns, D.B., 2011, Editorial: will the real neutrophil please stand up? Journal of leukocyte biology 90, 1039-41.

Acknowledgements

This work was supported by the Swedish Research Council (521-2009-3443), the King Gustav V Memorial Foundation, Gothenburg Medical Society, Ingabritt and Arne Lundgren's Research Foundation, the Gothenburg Rheumatism Association, and the Swedish state under the LUA/ALF agreement. The study sponsors had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the report; nor in the decision to submit the paper for publication.

Abbreviations

KRG, Krebs–Ringer phosphate buffer; OLFM4, olfactomedin 4; NGAL, neutrophil gelatinase-associated lipocalin; GFP, green fluorescent protein; MOI, multiplicity of infection;

Figure legends

Figure 1. Materials used for the creation of skin blisters

A handheld vacuum pump, attached to two suction chambers (A), is used to initiate the formation of skin blisters. The suction chambers are attached to the volar fore arm of a subject (B), ensuring negative pressure of 300-400 mmHg with a good seal. After approximately 1.45h, the suction chambers are removed and the blisters (C) are covered with plastic cups (D) and wrapped in elastic bandage to protect the blisters from damage.

Figure 2. Characterization of the inflammatory infiltrate

At different time points after blister formation, the blister fluid was incubated with a fluorophore-coupled antibody for the leukocyte antigen CD45 and analyzed by flow cytometry (A). Debris and cell fragments (CD45 negative and/or SSC low) were present already at T0 and excluded from further analyses. Three distinct leukocyte populations were observed at T6-T24, corresponding to neutrophils (PMN), monocytes (Mo), and lymphocytes (Ly). (B) The number of infiltrated leukocytes (CD45 positive) in the blister fluid is shown for the indicated time points to reflect the kinetics of leukocyte transmigration. Graph shows individual data points from a total of 5 donors sampled at different time points (C) The relative abundance of the different leukocyte subsets (specified in A) varied over time and is shown for T6-T24 as mean ± SEM from blisters drawn on 5 donors.

Figure 3. Viability of blister neutrophils

Viability of blister neutrophils was determined by Annexin V / To-Pro-3 staining and flow cytometry. Shown are samples from T6 (left) and T24 (right) blisters from one representative experiment.

Figure 4. Soluble inflammatory mediators in blisters

Blister fluids were analyzed for IL-1β, IL-6, IL-8, and TNF-α at the indicated time points (open squares) after blister formation by multiplex bead analysis. Shown are means ± SD from blisters of 6 donors and serum from three unrelated healthy donors (closed squares, for comparison) analyzed simultaneously. The lower detection limit for each cytokine is indicated by the dotted line.

Figure 5. Analysis of cell surface markers

The expression of surface markers CR3 and L-selectin on neutrophils (A) and monocytes (B) in T24 blister fluid (red histograms) and leukocytes isolated from peripheral blood (black histograms) from the same donor. Also shown for comparison is blood neutrophils after 20 min stimulation with TNF-α (blue histograms in A) to induce *in vitro* priming of cells. Blister cells display decreased L-selectin and increased CR3, indicative of *in vivo* priming. Shown are representative plots out of three independent experiments.

Figure 6. Analysis of intracellular proteins

Blood and blister neutrophils were immunostained for the specific granule proteins NGAL and OLFM4 and analyzed by FACS. (A) Histograms show isotype control (red) and NGAL (black) staining of the main populations from isolated blood leukocytes and blister fluid derived from the

same donor. (B) The dot plot (left) shows double staining of NGAL and OLFM4 in blister neutrophils of one representative donor out of three, depicting two distinct populations, one $NGAL^{high}$ $OLFM4^{high}$ and one $NGAL^{high}OLFM4^{low}$. The diagram (right) shows the percentage of OLFM4-positive neutrophils (based on NGAL-positivity) in blood and blister fluid from three different donors.

Figure 7. Phagocytosis of S. aureus *by blister phagocytes*

T6 blister fluid was incubated with GFP-expressing *S. aureus* (green) on ice or at 37^{o}C for 15 min at an MOI of 5 bacteria per phagocyte. (A) Flow cytometric analysis of the proportion GFP positive blister phagocytes, mean + SD is shown from 3 independent donors. (B) The images shown were obtained using an imaging flow cytometer, and depict representative cells incubated at the two different temperatures. The leukocyte marker CD45 (red) and nuclear stain DAPI (blue) were used to identify the phagocytes. BF=brightfield. The experiment was performed twice.

Supplementary figure 1. Soluble inflammatory mediators in blisters

Blister fluids were analyzed for IL-10, IL-12, IL-17, GM-CSF, IFN-γ, and IP-10 at the indicated time points after blister formation by multiplex bead analysis. Shown are means ± SD from blisters (open squares) of 6 donors and serum (closed squares, for comparison) from three unrelated healthy donors analyzed simultaneously. Where applicable, the lower detection limit for a cytokine analysis is indicated by dotted lines.

Figure 1. Davidsson *et al.*

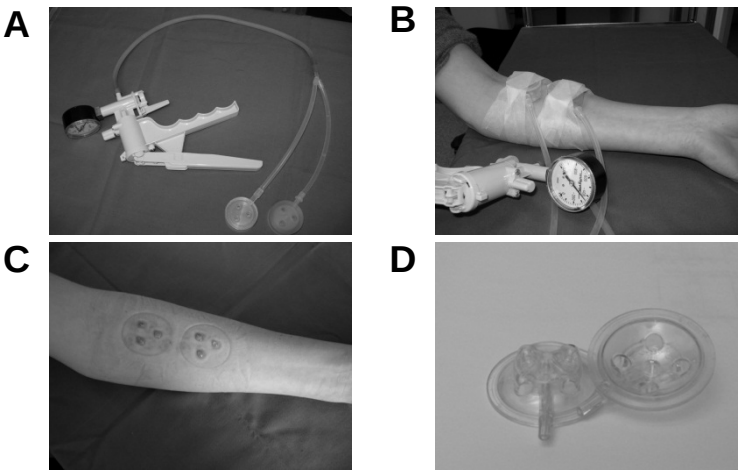


Figure 2. Davidsson *et al.*

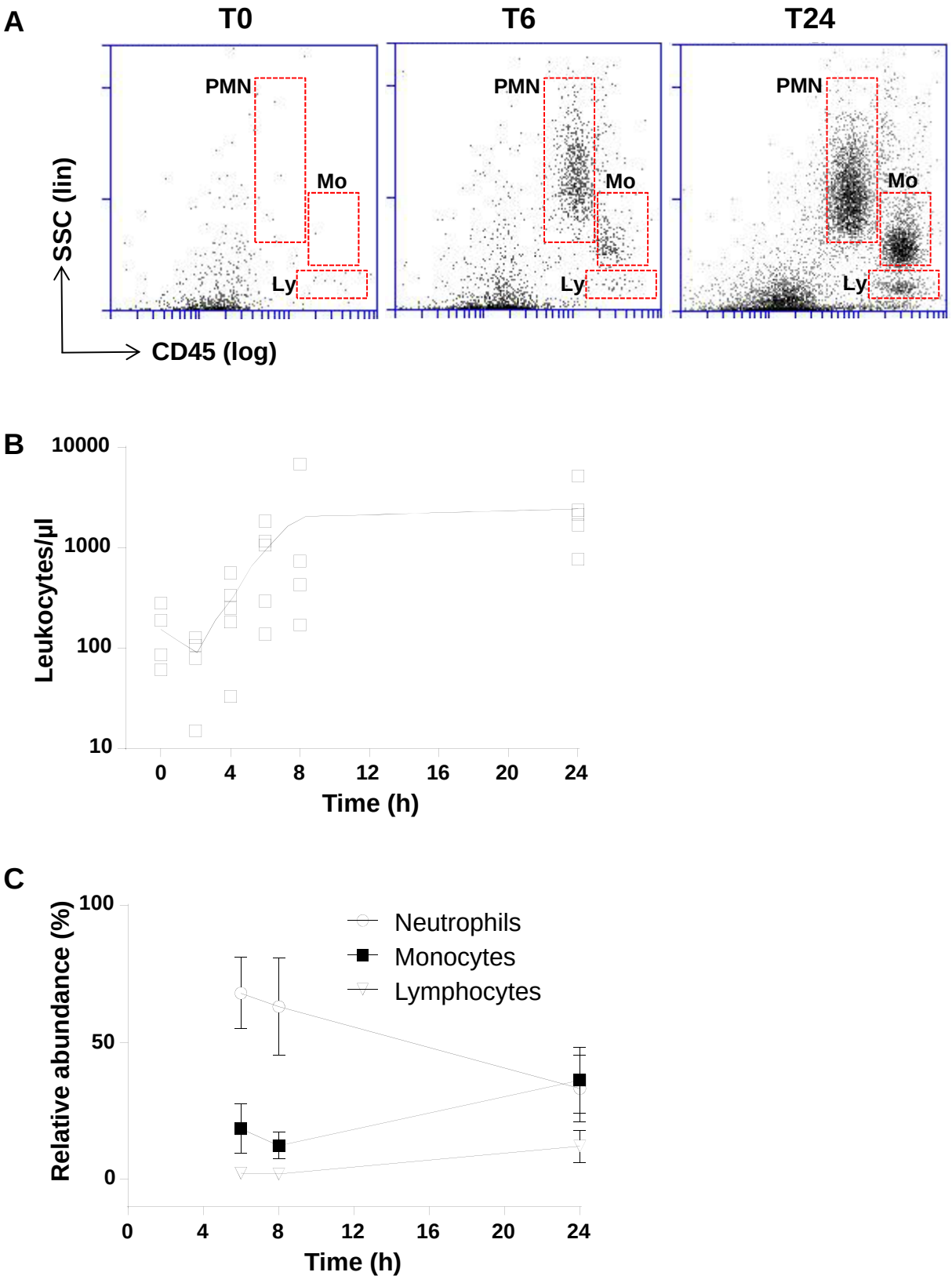


Figure 3. Davidsson *et al.*

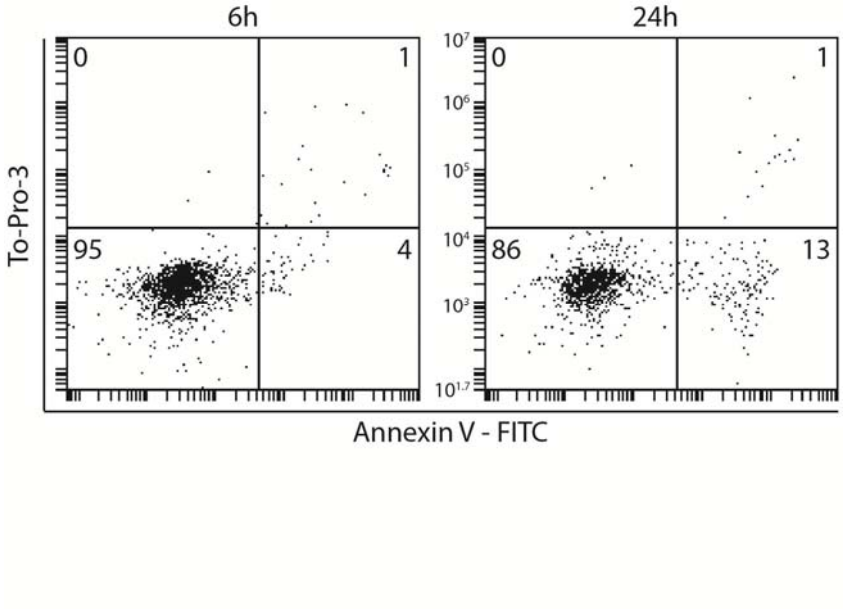
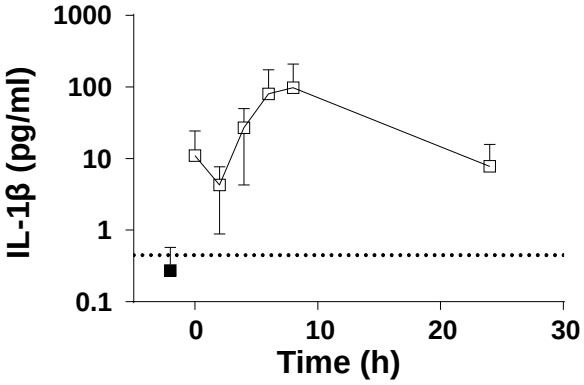
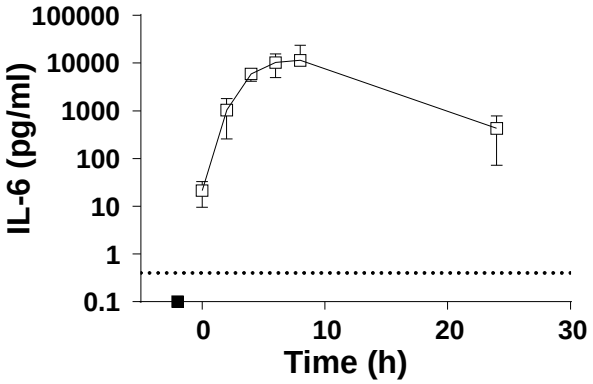
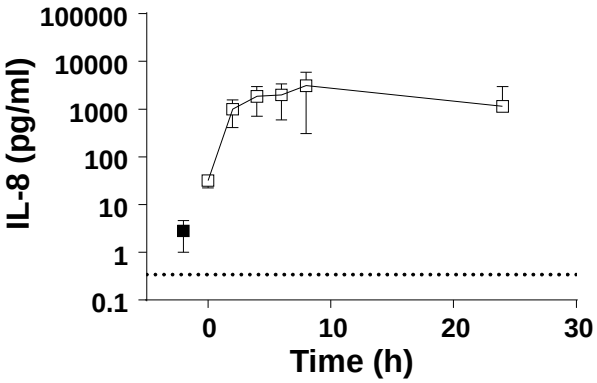


Figure 4. Davidsson *et al.*







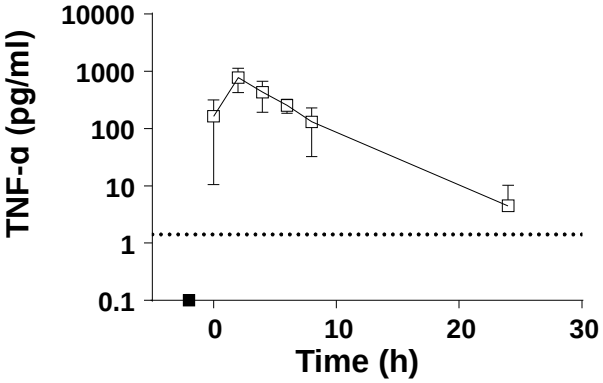


Figure 5. Davidsson *et al.*

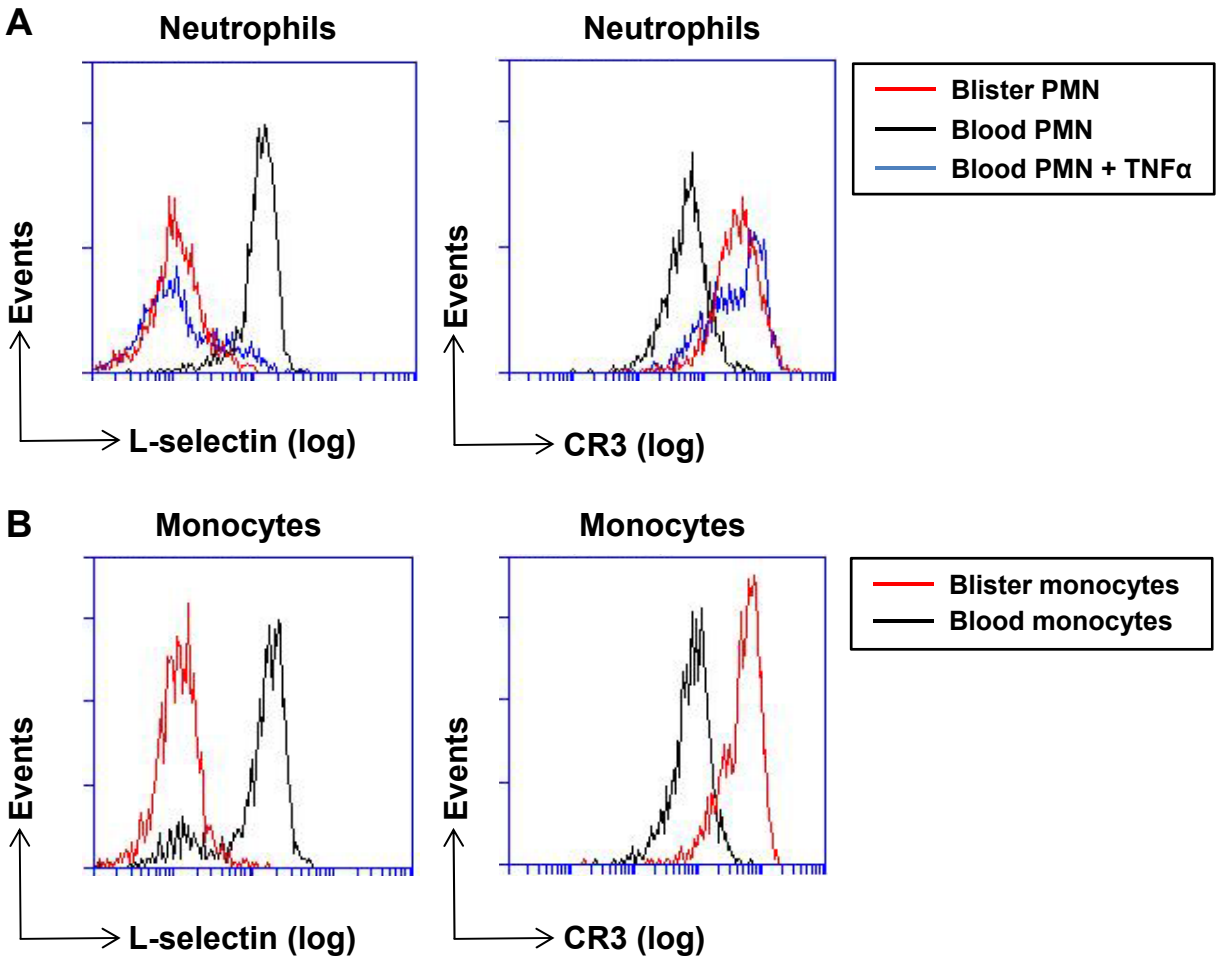


Figure 6. Davidsson *et al.*

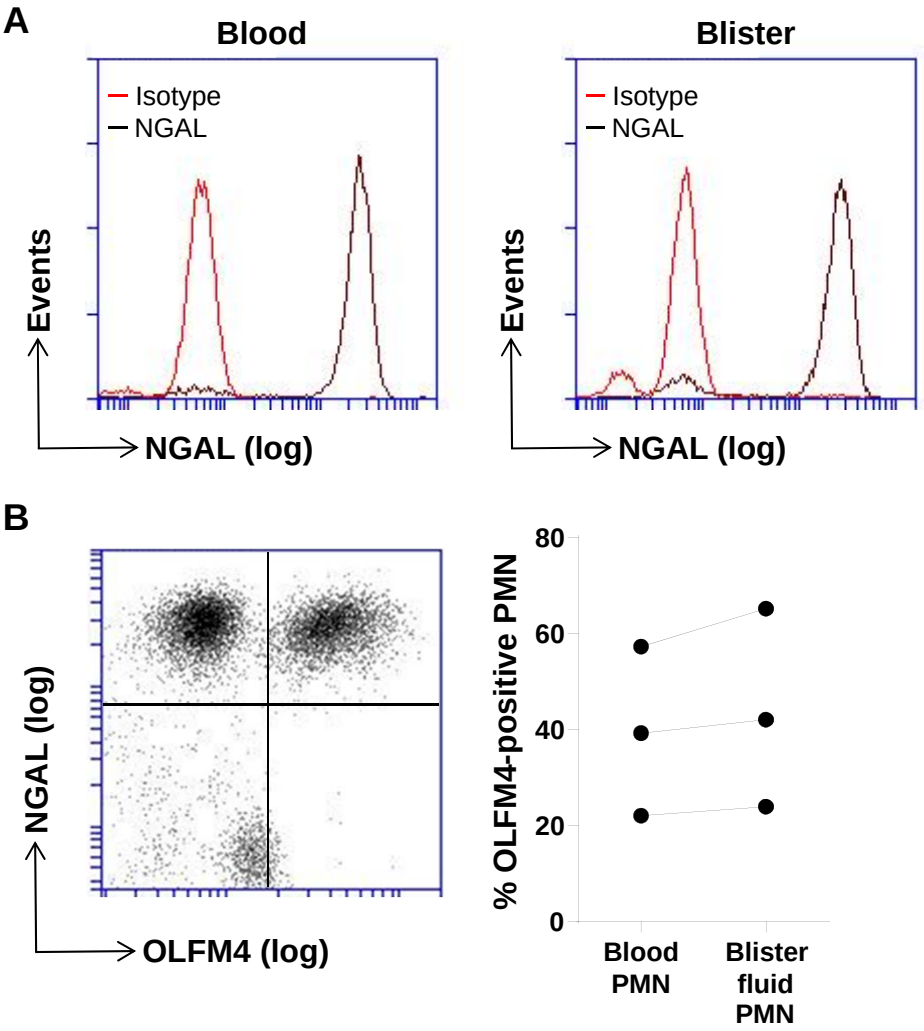
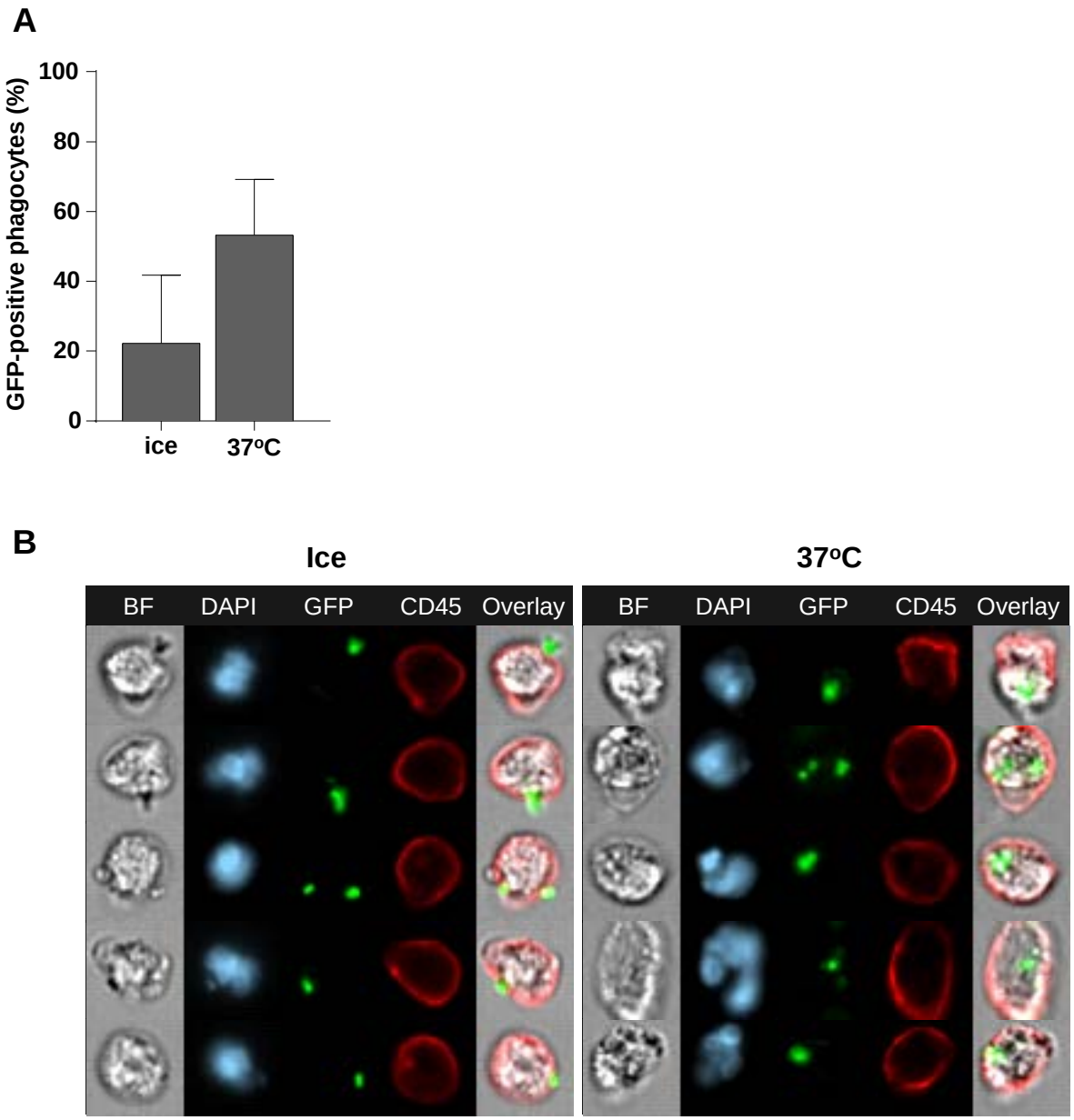


Figure 7. Davidsson *et al.*



Supplementary Figure 1. Davidsson *et al.*