Supporting Information

Nanomaterial Interactions with Human Neutrophils

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Evaluation of neutrophil phenotype and purity by flow cytometry

Any isolation procedure that separates primary cells from the *in vivo* environment risks altering cellular phenotype. Neutrophils, non-proliferative and primed to respond dramatically to external stimuli, are especially susceptible to rapid activation, apoptosis and/or cell death as a result of improper handling. We selected a well-established[1] four-step isolation procedure from fresh citrate-stabilized, room temperature buffy coats that involves: 1) Dextran sedimentation of red blood cells (RBCs), 2) Slow pelleting to remove platelets, 3) Density gradient centrifugation to remove lymphocytes and monocytes, and 4) Brief hypotonic lysis to remove residual RBCs. Immediately following isolation, we stained cells to evaluate purity, viability, and phenotype by flow cytometry (FC), as outlined in Table 1.

Table S1 | Neutrophil Markers for Flow Cytometry

Marker	Description	Apoptotic Neutrophils	Non-Activated Neutrophils	Activated Neutrophils
CD45	Pan-leukocyte plasma membrane receptor.	+	+	+
CD16	FcγRIII low-affinity receptor on mature neutrophils[2]. Differentiates between neutrophils and eosinophils in a population of purified human granulocytes.	Decreased	+	+
CD62L/ L-Selectin	Adhesion molecule on surface of leukocytes; cleaved from the surface of neutrophils following activation[3].	N/A	+	Decreased
LIVE/DEAD™	Thermo Fisher cell viability assay uses fluorescence-based Molecular Probes™ to identify dead cells	-	-	-
Side scatter	Higher for granulocyte populations than monocytes or lymphocytes – reflects the increased internal complexity of granule-loaded cells.	Hi	Hi	Hi

Briefly, the characteristic separation of granulocytes from monocytes and lymphocytes based on their forward scatter vs. side scatter profile was used to gate the population of likely granulocytes. Granulocytes, due to the greater internal complexity of their dense granule compartments, exhibit higher side scatter than other leukocytes. Typical FC results show that 90-95% of events consist of single granulocytes (Figure S1a), with the rest consisting primarily of residual monocytes or lymphocytes. The amine-reactive Live/Dead Aqua dead cell stain generates a ~50-fold increase in fluorescence in cells with compromised membrane integrity, and was included to assess cell viability. Of FSC/SSC gated granulocytes, >99% experienced no loss of membrane integrity due to isolation and preparation for FC analysis (Figure S1b).

To distinguish eosinophils, a known contaminant in our isolation procedure, we stained cells with antibodies to the cell surface markers CD45 (a pan-leukocyte marker) and CD16, (a low-affinity IgG receptor). Neutrophils are $CD45^{+}CD16^{+}$, whereas eosinophils are $CD45^{+}CD16^{-}$. Plotting CD45 vs. CD16 intensity clearly shows two unique cell populations, of which ~1.5% are eosinophils and the rest are neutrophils (Figure S1c i and ii+iii, respectively). This 60:1 ratio compares well with the typical relative abundance of neutrophils and eosinophils in human blood. CD16, in particular, was selected from among several potential neutrophil-specific cell surface receptors on the basis of previous reports describing CD16 shedding as a marker for neutrophil apoptosis[2]. Setting a conservative gate to distinguish $CD16^{hi}$ from $CD16^{lo}$ states shows that ~90% of neutrophils are non-apoptotic post-isolation.

Among non-apoptotic neutrophils, we also assessed activation with L-selectin (CD62L), a cell surface receptor known to be rapidly shed from *ex vivo* neutrophils shortly after activation[3]. To determine appropriate gating, we exposed neutrophils to various secretagogues (the chemotactic bacterial peptide n-formyl-Met-Leu-Phe [fMLP], the calcium ionophore ionomycin

and the protein kinase C activator phorbol 12-myristate 13-acetate [PMA])[4] and measured CD62L expression levels (Figure S1d). The most potent stimulant, a combination of 1 μM ionomycin and 50 nM PMA, reduced mean CD62L surface expression levels by a factor of ~25. We chose to set our gate at a fluorescence level below which 99.9% of neutrophils co-stimulated by ionomycin and PMA may be found (Figure S1e). Based on this gating strategy, only ~5% of non-apoptotic neutrophils can be considered activated; however, they retain their ability to become activated, as between 80-99.9% of neutrophils shed CD62L in the presence of known stimulants (Figure S1f). Taken together, these results indicate that our isolation protocol produces live, reasonably pure, and predominantly non-apoptotic neutrophils, with minimal activation.

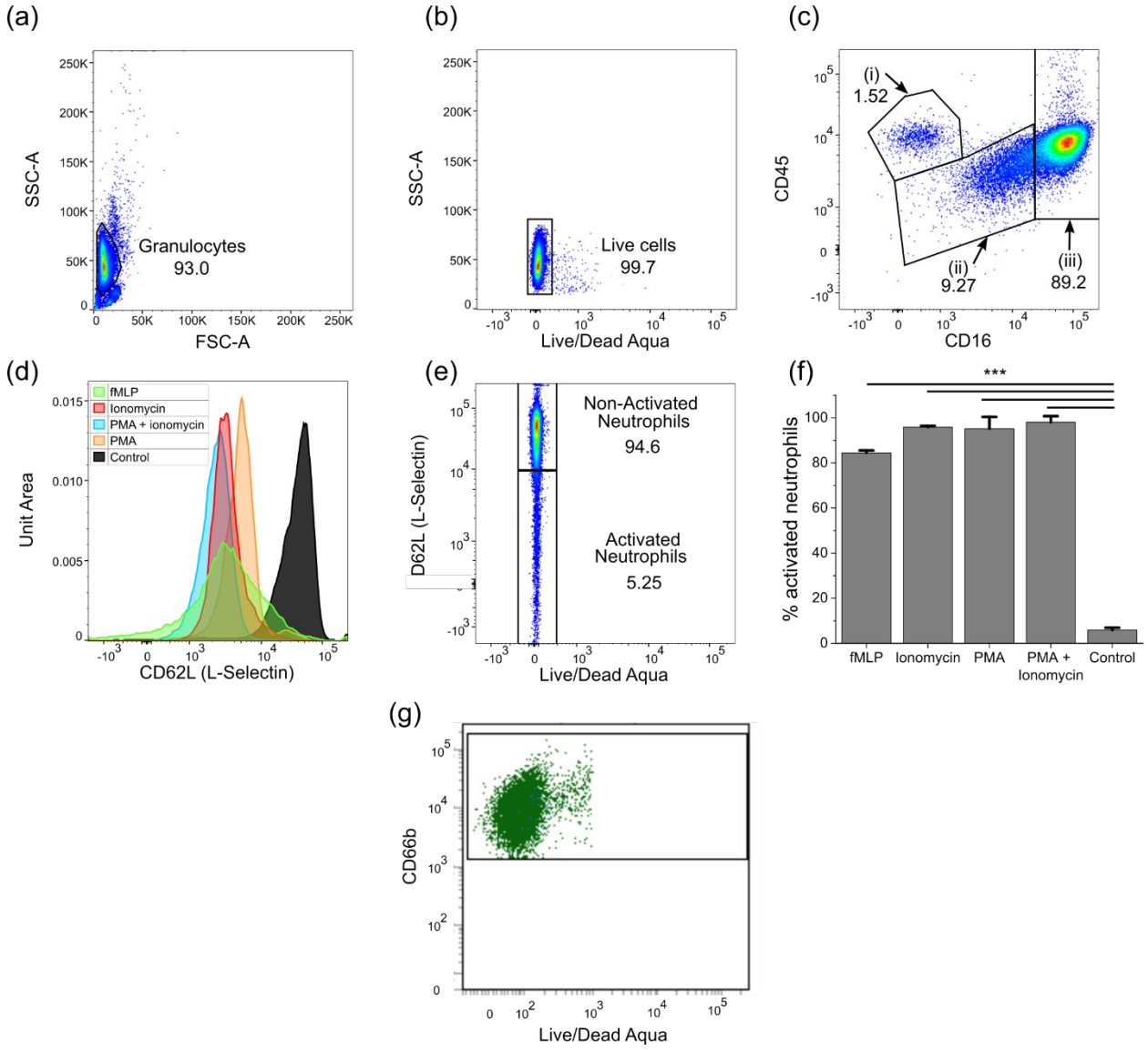

Figure S1 | The ex vivo neutrophil isolation procedure leads to a reasonably pure neutrophil population while minimizing apoptosis and activation. (a) Flow cytometry forward scatter vs. side scatter profile for as-isolated neutrophils. Frequency of cells gated as granulocytes shown in percent. (b) Results of dead cell staining for the granulocyte-gated population. Frequency of cells gated as live shown in percent. (c) CD45 vs. CD16 expression in cells gated as live granulocytes. Region (i) is thought to be eosinophils ($CD45^{+}CD16^{-}$), region (ii) is labeled as apoptotic

neutrophils ($CD45^+CD16^{lo}$), and region (iii) is labeled as non-apoptotic neutrophils ($CD45^+CD16^{hi}$). Frequencies shown in percent. (d) CD62L expression is significantly higher in untreated vs. stimulant-treated neutrophils, indicating that (i) the isolation procedure does not activate neutrophils and (ii) the neutrophils retain their ability to become activated *ex vivo*. (e) FC gating to distinguish between activated and non-activated neutrophils. Gate location is set at the point of CD62L below which 99.9% of PMA+ionomycin stimulated neutrophils would fall. (f) Percentage of cells called activated for each stimulant based on the gating strategy described in Figure S1e. (g) 99.4% of cells gated as single, live granulocytes were $CD66b^+$. In a healthy human subject this nominally includes both eosinophils and neutrophils. *** indicates $p < 0.0001$. Error bars shown are standard deviations, n = 4 (with ~10K cells per repetition).

Supplementary References

(1) Nauseef, W. M. Isolation of Human Neutrophils from Venous Blood. *Methods Mol. Biol.* (N. Y.) **2007**, *412*, 15–20. doi: 10.1007/978-1-59745-467-4_2

(2) Dransfield, I.; Buckle, A. M.; Savill, J. S.; McDowall, A.; Haslett, C.; Hogg, N. Neutrophil Apoptosis Is Associated with a Reduction in CD16 (Fc Gamma RIII) Expression. *J. Immunol.* **1994**, *153* (3), 1254–1263.

(3) Griffin, J. D.; Spertini, O.; Ernst, T. J.; Belvin, M. P.; Levine, H. B.; Kanakura, Y.; Tedder, T. F. Granulocyte-Macrophage Colony-Stimulating Factor and Other Cytokines Regulate Surface Expression of the Leukocyte Adhesion Molecule-1 on Human Neutrophils, Monocytes, and Their Precursors. *J. Immunol.* **1990**, *145* (2), 576–584.

(4) Udby, L.; Borregaard, N. Subcellular Fractionation of Human Neutrophils and Analysis of Subcellular Markers. *Methods Mol. Biol. (N. Y.)* **2007**, *412*, 35–56. doi: 10.1007/978-1-59745-467-4_4