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Heparin Reduces Nonspecific Eosinophil Staining Artifacts in Mass Cytometry Experiments

Adeeb H. Rahman[1,2,3,*], **Leticia Tordesillas**[3,4], and **M. Cecilia Berin**[3,4]

[1]Human Immune Monitoring CoRE, Icahn School of Medicine at Mount Sinai, New York, New York, 10029

[2]Department of Genetics and Genomics, Icahn School of Medicine at Mount Sinai, New York, New York, 10029

[3]Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, New York, 10029

[4]Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, New York, 10029

Abstract

The analysis of heterogeneous cell samples by mass cytometry (CyTOF) relies on the assumption that metal labeled antibodies accurately bind to their target antigens. We report a previously unappreciated experimental artifact of non-specific antibody binding by eosinophils during intracellular CyTOF analysis of human whole blood samples. We hypothesized that this non-specific binding results from a charge-based interaction between the metal-labeled antibodies and highly cationic proteins found in eosinophillic granules and found that this non-specific staining artifact could be reduced to background levels with a simple blocking protocol using heparin as a competing anionic protein. This protocol eliminates a potential source of erroneous data interpretation in all experiments involving intracellular staining of human whole blood samples, and allows accurate assessment of dynamic changes in intracellular proteins in eosinophils by CyTOF.

Keywords

eosinophil; granulocyte, nonspecific antibody binding; whole blood; heparin; CyTOF; mass cytometry

MASS cytometry is an advanced technology for high dimensional single cell analysis, in which heterogeneous cell populations are labeled with antibodies conjugated to stable metal isotopes and analyzed by time-of-flight mass spectroscopy using the CyTOF platform (1). Metal-labeled antibodies can be used to detect both surface and intracellular antigens, and a typical CyTOF experiment allows the simultaneous analysis of over 35 parameters in a single sample. This allows for a detailed dissection of the cellular heterogeneity in a sample using traditional manual gating approaches and a growing number of automated clustering and population identification algorithms.

[*]Correspondence to: Adeeb Rahman, 1470 Madison Ave, s6-103, New York, NY, 10029. adeeb.rahman@mssm.edu.

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Mass cytometry data typically exhibits lower levels of background signal than flow cytometry data, due to the fact that the stable metal isotopes used are present at very low abundance in biological samples, and there is minimal signal spillover between distinct detection channels. However, the validity of CyTOF data relies on the assumption that the isotopic abundance of a given tag is an accurate reflection of the abundance of the respective target protein, as would be expected when the metal-labeled antibodies bind specifically to their intended target epitopes. Therefore, as with traditional flow cytometry, nonspecific antibody binding presents a technical challenge and can lead to erroneous interpretation of results.

It is generally recognized that nonspecific staining may occur in both flow and mass cytometry experiments when antibodies cross react with secondary epitopes, or bind to Fc receptors expressed on the surface of some cell types. Here, we report another prevalent but previously unappreciated form of non-specific antibody binding by eosinophils during intracellular CyTOF analysis of fixed whole blood samples, which we believe occurs due to charge-based interactions between anionic metal-polymer-conjugated antibodies and cationic proteins that are highly expressed in eosinophils (2). The unexpected staining patterns that result from this nonspecific binding can confound accurate data analysis; while unanticipated data may be “gated out” and excluded, this limits truly comprehensive and unbiased analyses of complex samples, which is one of the major advantages offered by highly multiparametric CyTOF experiments. This may account for why eosinophils, despite being an important and relatively prevalent circulating immune population, have not been described in previous CyTOF studies that have evaluated protein phosphorylation across immune populations in whole blood (3›4). Here, we describe that this non-specific eosinophils staining can be successfully reduced to background levels using heparin blocking, thereby eliminating a major potential artifact in mass cytometry experiments and improving the accuracy and comprehensiveness of unbiased population discovery and intracellular protein measurements across cell types in heterogeneous samples.

Materials and Methods

Subjects and Samples

Blood samples were obtained from food allergic pediatric donors being seen for routine care at the Jaffe Food Allergy Institute between January and May 2014, under a protocol approved by the Institutional Review Board of the Icahn School of Medicine at Mount Sinai (HSM 11–00645, PI Hugh Sampson). Blood was obtained in heparinized vacutainer tubes and used for staining within ~3 h from the time of blood draw.

Antibody Staining

All the antibodies used in this study were either purchased pre-conjugated from Fluidigm or were conjugated using X8 MaxPar conjugation kits according to the manufacturer’s protocol (Table 1). Titrated antibodies against CD45, CCR3, CD294, and CD123 were added to whole blood prior to fixation to enable accurate identification of eosinophils. Additional antibodies to distinguish other cell populations and evaluate nonspecific antibody binding were added either prior to fixation, or following treatment with BD Phosflow Lyse/Fix

buffer (BD Biosciences) and permeabilization with 0.1% Triton X-100, as indicated in the figure legends. After washing, the samples were then incubated with 0.125 nM Ir nucleic acid intercalator (Fluidigm) to enable cell identification based on DNA-content, and stored in PBS with freshly diluted 2% formaldehyde (Electron Microscopy Sciences) until acquisition.

Heparin Blocking

Heparin sodium salt (Sigma, H3393) was diluted as a 10 KU mL^{-1} stock in PBS and stored at 4°C. Heparin blocking was performed by resuspending fixed/permeabilized blood samples in 50 μL of PBS with 0.2% BSA and 0, 3, 10, 33, 100, or 333 U mL^{-1} heparin as indicated in the figure legends. Sample were incubated for 20 min at room temperature before the addition of MaxPar antibodies diluted in an additional 50 μL of PBS with 0.2% BSA to give a total staining volume of 100 μL.

Whole Blood Stimulation for Phospho-Protein Staining

Whole blood was first stained with a panel of antibodies against cell surface antigens to enable immune cell discrimination and then divided into three aliquots. One was left as an unstimulated control, while the other two received 50 ng mL^{-1} granulocyte-macrophage colony stimulating factor (GM-CSF) and 50 ng mL^{-1} Interleukin-2 (IL-2) (PeproTech), respectively. The samples were incubated for 10 min at 37°C and then treated with BD Phosflow Lyse/Fix buffer according to the manufacturer's instructions. The samples were then permeabilized with ice-cold methanol, washed and stained for intracellular p-STAT5 expression with and without prior heparin blocking, as described above.

Data Acquisition and Analysis

Immediately prior to acquisition, the samples were washed once with PBS, once with deionized water and resuspended at a concentration of 600,000 cells mL^{-1} in water containing a 1/20 dilution of EQ 4 element beads (Fluidigm). Following routine autotuning according to the manufacturer's recommendations, the samples were acquired on a CyTOF2 mass cytometer (Fluidigm) at a flow rate of 0.045 mL min^{-1}. For quality control, the acquisition event rate was maintained under 400 events s^{-1}, and the EQ beads were confirmed to have a median Eu151 intensity of over 1000 to ensure appropriate mass sensitivity. The resulting FCS files were normalized using the bead-based normalization tool in the CyTOF2 software and uploaded to Cytobank for analysis. Cells events were identified as Ir191/193 DNA+ Ce140-events, and doublets were excluded on the basis of higher DNA content and longer event length. Of note, we observed subtle differences in the median Ir191/193 signal intensity between granulocyte and lymphocyte populations, with the highest expression found in eosinophils. Thus, the Ir191/193 cutoff for optimal singlet/doublet discrimination for eosinophils was slightly different than for lymphocytes (data not shown).

Major immune populations were identified either by subjective manual gating on biaxial plots (as shown in Fig. 1) or by using viSNE, a dimensionality-reducing algorithm based on t-Distributed Stochastic Neighbor Embedding (t-SNE), which allows multi-dimensional single cell expression data to be mapped to two-dimensional space (5). The resulting viSNE

map resembles a conventional biaxial cytometry plot in which the spatial position of the cells reflects their multidimensional relationships. The expression patterns of canonical cell surface markers were iteratively analyzed as a third dimension to identify putative population regions on the viSNE map (as shown in Fig. 3). It should be noted that in the absence of heparin blocking, the accurate resolution of eosinophils as a consistent population on a viSNE map was only possible when using antibodies added prior to blood fixation due to the non-specific antibody binding post fixation.

Nonspecific antibody staining by eosinophils was visualized based on the staining intensity of antibodies specific to cell surface antigens that are not expressed by human eosinophils (e.g., CD3, CD4, CD8, CD22, CD16, and murine CD45, as indicated in the figure legends). To account for the bimodal non-specific staining distribution, the heparin-induced reductions in nonspecific staining were visualized and quantified based on the reduction in 90th percentile staining intensity on the total eosinophil population. An overall “nonspecific staining index” was calculated as the average of the 90th percentile staining intensity for all the antibodies added to the sample post fixation, normalized to their respective intensity in the absence of heparin.

Results and Discussion

Blood Eosinophils Nonspecifically Stain WITH CyTOF Antibodies Following Fixation and Permeabilization

Eosinophils are traditionally distinguished by their high side-scatter (SSC) profiles by flow cytometry. While mass cytometry does not permit the measurement of light scattering parameters, eosinophils were readily identifiable in whole blood samples as a CD66-expressing granulocyte population with low CD16 expression and high expression of the prostaglandin receptor CD294 (CRTH2) and chemokine receptor CCR3 (6) (Fig. 1A). Basophils also share high expression of CRTH2 and CCR3 with eosinophils, but were easily distinguishable based on their high expression of the IL-3Ra chain, CD123, which eosinophils lack (data not shown). We observed clear positive staining patterns for CD294 and CCR3 when these antibodies were added to fresh whole blood, but the positive populations were no longer resolvable following fixation, suggesting that the binding epitopes of these antibodies are sensitive to formaldehyde fixation (data not shown).

When multiparametric mass cytometry antibody staining was performed on whole blood prior to fixation, eosinophils exhibited the predicted staining profile, with low expression of markers that are considered to be specific to other immune cell lineages, such as B cells (CD22) and T cells (CD3) (Fig. 1B). However, when mass cytometry antibodies were added to whole blood after fixation and erythrocyte lysis with commercial Lyse/Fix buffers, we found that eosinophils exhibited high levels of staining in all antibody channels and typically exhibited a bimodal staining distribution, with a subset of eosinophils showing higher non-specific binding of all antibodies. The non-specific staining was further exacerbated when the antibodies were added following plasma membrane permeabilization, particularly with Triton X-100. The broad eosinophil positivity for multiple antibodies suggests an antigenindependent, epitope-non-specific binding mechanism, which was further supported

by the finding that human eosinophils also stained positively with antibodies specific to mouse CD45 following fixation and permeabilization (Fig. 1C).

A distinguishing feature of eosinophils are their cytoplasmic crystalloid granules, which contain high levels of arginine-rich cationic proteins, including eosinophil cationic protein (ECP), major basic protein (MBP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (2). It has long been recognized that these proteins can result in non-specific binding to anionic fluorochromes, such as FITC, leading to false-positive signals in immunofluorescence labeling (7,8). CyTOF MaxPar antibodies are labeled with lanthanide isotopes using proprietary X8 or DN3 metal-binding polymers, and the metal-loaded polymers retain a net-anionic charge (Fluidigm, personal communication). We therefore hypothesized that the nonspecific eosinophil staining detected by CyTOF was also due to an ionic interaction between the cationic eosinophils granules, and the anionic antibodies. While we have observed nonspecific staining with antibodies conjugated to most of the commonly used lanthanide isotopes (Fig. 1 and data not shown), we have noted differences in the relative nonspecific staining intensity with different antibodies. This could relate to differences in the specific polymer used, the efficiency of metal loading per polymer, or the number of polymers bound per antibody, all of which may be expected to alter the overall charge of the antibody–polymer complex, which in turn may affect the strength of the ionic interactions.

This nonspecific binding would only be expected to occur when disruptions of the plasma membrane would permit interactions between the antibodies and the granules, which would account for the differences in staining patterns with antibodies pre- and post-fixation/permeabilization. Differences in the levels of non-specific antibody binding that we have observed between individuals may therefore reflect interindividual heterogeneity in eosinophil granule content (9). Furthermore, the bimodal nonspecific staining distribution that we typically observe suggests additional heterogeneity of cationic granule content within the circulating eosinophil population, perhaps reflecting differences in the state of maturation, activation or degranulation (10).

Heparin Treatment Effectively Prevents Nonspecific Eosinophil Antibody Binding in a Dose-dependent Manner

If non-specific binding occurs due to a charge-based interaction between the eosinophils and the MaxPar antibodies, we hypothesized that this interaction might be prevented by blocking the cationic charges with an alternative anionic compound prior to addition of the antibodies. Heparin is a complex polysaccharide that is highly anionic by virtue of its numerous sulfate groups. ECP has previously been shown to bind to heparin with high affinity through both electrostatic interaction and specific structural interactions with its RNase catalytic site, and treatment with heparin can limit ECP-mediated damage to pathogens and to epithelial cells (11,12). Heparin has also been reported to bind to MBP and does so with much higher binding affinity than other anionic gylcosaminoglycans such as chondroitin sulfate or hyaluronic acid (13). In addition to its high negative charge density, heparin is also an affordable and readily available reagent, which made it a good candidate for a potential anionic blocking agent.

We performed a heparin dose titration as a brief blocking step following whole blood fixation/permeabilization, immediately prior to addition of the MaxPar antibodies. We found that heparin effectively blocked nonspecific eosinophil antibody binding in a dose-dependent fashion (Fig. 2). A significant inhibition of nonspecific staining was observed even with 3 U mL^{-1} heparin, with a more dramatic effect apparent in the Triton-permeabilized samples (Fig. 2C), which exhibited higher levels of nonspecific staining in the absence of heparin. We noted some variability in the dose response to heparin blocking between individuals at lower concentrations of heparin, but nonspecific staining was effectively reduced to the level of background in all samples with 100 U mL^{-1} heparin (Figs. 2D and 2E).

Importantly, while heparin treatment inhibited nonspecific antibody binding, it did not compromise specific antibody recognition, as evidenced by the lack of change in signal intensity for positive antibody staining in response to increasing heparin concentrations (e.g., CD22 expression on B cells, CD3 expression on T cells or CD16 expression on neutrophils). Furthermore, heparin did not abrogate antigen-specific CD66a staining on eosinophils. Overall, these findings suggest that heparin blocking offers an effective means to eliminate non-specific antibody binding to eosinophils.

Heparin Blocking Allows Accurate Assessment of Intracellular Protein Phosphorylation Patterns across Immune Populations in Human Whole Blood

To demonstrate the utility of this heparin blocking protocol in typical CyTOF experiments, we performed an experiment to evaluate patterns of protein phosphorylation across whole blood immune populations in response to cytokine stimulation. Whole blood samples were stained with a panel of antibodies allowing the discrimination of major immune cell subsets using viSNE (5), a visualization tool that allows high-dimensional cytometry data to be mapped and represented in two-dimensional space (Fig. 3A). The blood samples were either left unstimulated, or stimulated with GM-CSF or IL-2; two cytokines that both induce signaling via STAT5 phosphorylation but exhibit their primary effects on different immune cell types due to distinct cytokine receptor distributions.

In the absence of heparin blocking, we found that eosinophils appeared to express very high levels of pSTAT5 even in the absence of stimulation (Fig. 3B). This high basal expression precluded the ability to resolve dynamic changes in STAT5 phosphorylation in response to GM-CSF, a cytokine that is expected to act on eosinophils, and persisted in the presence of IL-2, a cytokine that is not expected to act on eosinophils. Heparin blocking completely eliminated this high apparent pSTAT5 signal in unstimulated samples, and permitted the accurate detection of pSTAT5 upregulation in eosinophils in response to GM-CSF, but not in response to IL-2, consistent with the expected actions of these cytokines (Fig. 3C). Interestingly, we noted that heparin blocking also reduced background pSTAT5 intensity in neutrophils in this experiment, though to a far smaller extent than in eosinophils. This is consistent with the fact that neutrophils have also been shown to express cationic granule proteins though at levels ~100-fold lower than eosinophil, which suggests that heparin may offer additional benefits in reducing non-specific antibody staining in other cell types (2,14). The absence of the high non-specific staining intensity in eosinophils also allowed for viSNE visualization scaling that better reflected the true dynamic range of changes in

pSTAT5 intensity. Thus, heparin blocking eliminates false-positive staining and permits a more accurate analysis of the distribution and dynamic changes in intracellular protein expression by CyTOF.

Conclusions

We have found that blood eosinophils non-specifically bind to MaxPar antibodies when stained post fixation/permeabilzation, and these false positive signals complicate accurate population identification, and lead to erroneous interpretation of results when evaluating the distribution of intracellular proteins in CyTOF experiments. This nonspecific staining appears to be due to a charge-based interaction between highly cationic eosinophil granule proteins and the anionic MaxPar antibodies, and can be effectively blocked by pretreating fixed and permeabilized cells with heparin prior to the addition of intracellular staining antibodies. Heparin treatment does not appear to have a detrimental effect on specific antibody staining, and therefore permits the accurate measurement of intracellular protein expression in eosinophils, while also eliminating non-specific eosinophil antibody binding as a potential complicating artifact. Given the ready availability and low cost of heparin as a reagent, this approach should be widely applicable as a standard step in all mass cytometry experiments involving post-permeabilization intracellular antibody staining.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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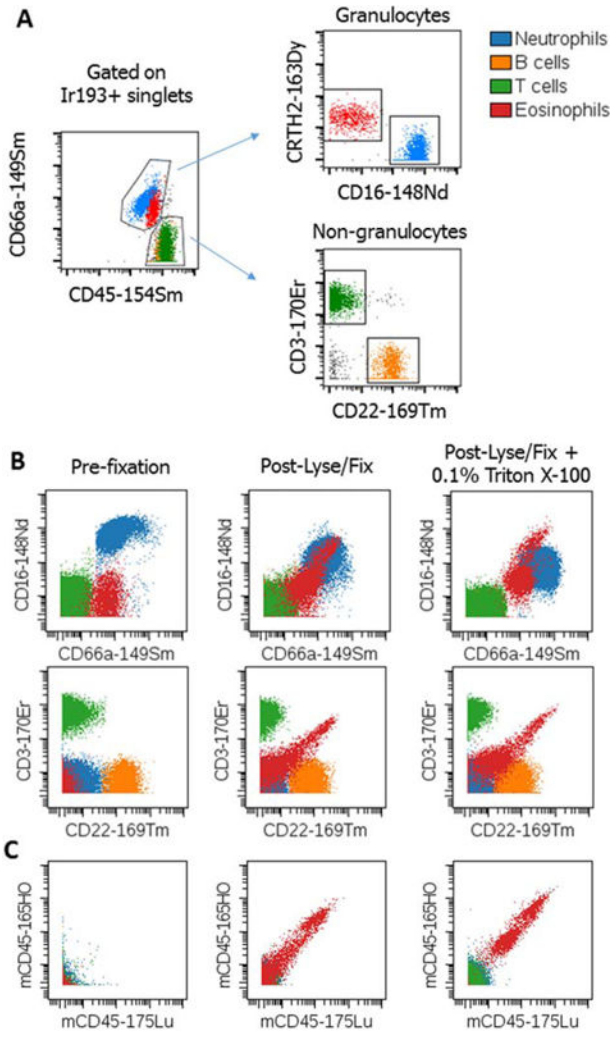

Figure 1.

Eosinophils non-specifically bind to antibodies added post-fixation/permabilization. (**A**) Fresh human whole blood can be stained with a combination of antibodies allowing clear identification of neutrophils, eosinophils, B cells and T cells by mass cytometry. (**B**) When antibodies are added to whole blood prior to fixation, eosinophils express CD66a, but are negative for CD16, CD3, and CD22 as is expected, however, when antibodies are added following fixation or permeabilization, eosinophils show positive staining for all the antibodies with a bimodal staining distribution. (**C**) Eosinophils also exhibit non-specific staining with antibodies specific to mouse CD45.

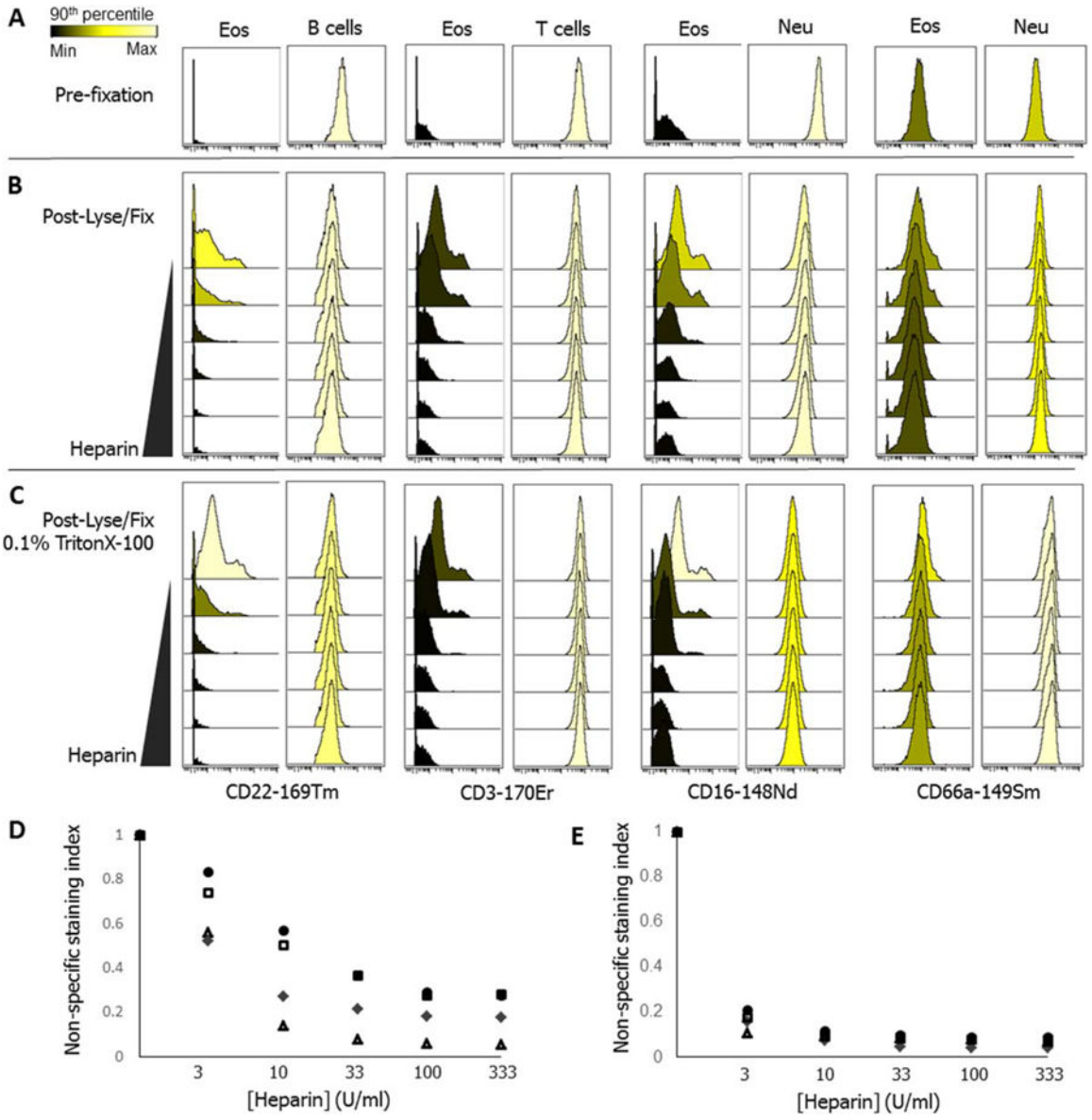

Figure 2.

Heparin blocking prevents non-specific eosinophil antibody staining. Fresh blood samples were stained with antibodies against CD45, CD66a, CRTH2, CCR3, and CD123 to allow unambiguous identification of eosinophils as in Figure 1. In contrast to their expression profile in fresh blood (**A**), following lysis/fixation (**B**) and Triton X-100 permeabilization (**C**), eosinophils show elevated staining for CD22, CD3, CD16 antibodies with a bimodal positive staining pattern. This nonspecific staining is reduced to background levels by blocking with increasing concentrations of heparin (3–333 U mL^{-1}), while positive CD66a staining is retained. Histograms are colored to show 90th percentile signal intensity, scaled independently for each parameter. The variation in heparin dose response is shown for four individual donors following lysis/fixation (**D**) and Triton permeabilization (**E**). The nonspecific staining index was calculated as the average of the 90th percentile staining

intensity for all the antibodies added post fixation (CD22, CD3, CD4, CD8, and CD16), normalized to their respective intensities in the absence of heparin.

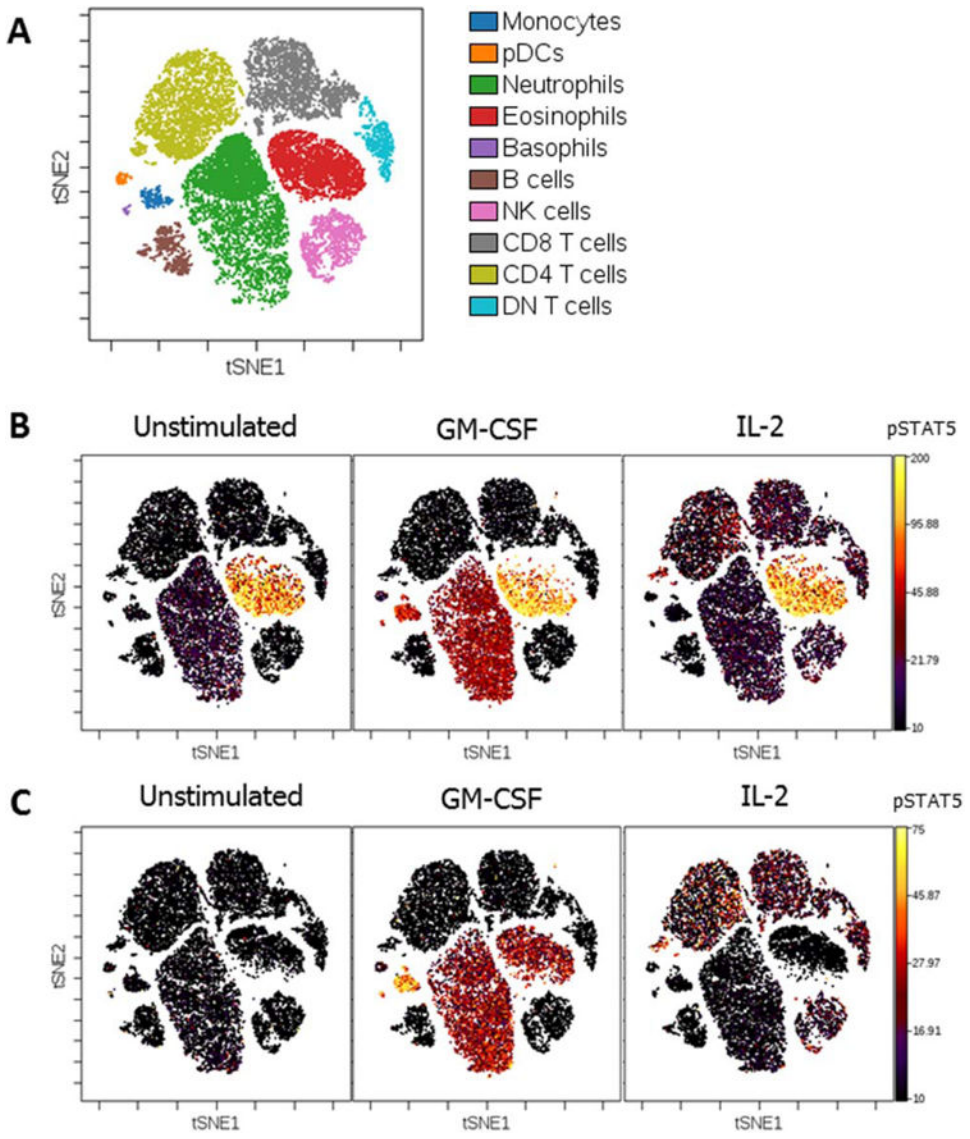

Figure 3.

Heparin blocking allows accurate assessment of intracellular protein phosphorylation. (**A**) Fresh whole blood was stained with a panel of antibodies against canonical cell surface markers, allowing the identification and visualization of major immune cell subsets by viSNE analysis. Stained blood samples were stimulated with GM-CSF or IL-2 and stained for intarcellular pSTAT5 expression. The distribution of pSTAT5 intensity across cell subsets on the viSNE map is shown without (**B**) and with (**C**) heparin blocking.

Table 1

Antibody information

ANTIBODY TARGET	CLONE	METAL TAG	SOURCE
CD19	H1B19	142Nd	Fluidigm
CD4	RPA-T4	145Nd	Fluidigm
CD8	OKT-8	146Nd	eBioscience
CD16	3G8	148Nd	Fluidigm
CD66a	CD66a-B1.1	149Sm	Fluidigm
pSTAT5	47	150Nd	Fluidigm
CD123	6H6	151Eu	Fluidigm
CD45	HI130	154Sm	Fluidigm
CD11c	Bu15	159Tb	Fluidigm
CD14	M5E2	160Gd	Fluidigm
CD294/CRTH2	BM16	163Dy	Fluidigm
CCR3	5E8	164Dy	Biolegend
CD22	HIB22	169Tm	Biolegend
CD3	UCHT1	170Er	Fluidigm
CD56	B159	173Yb	BD Biosciences
HLA-DR	L243	174Yb	Fluidigm

Non-Fluidigm sourced antibodies were conjugated in-house using Fluidigm's MaxPar X8 conjugation kits.