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In-depth phenotypic characterization of reticulocyte maturation using mass cytometry

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ABSTRACT

Progress towards an in-depth understanding of the final steps of the erythroid lineage development is paramount for many hematological diseases. We have characterized the final stages of reticulocyte maturation from bone marrow to peripheral blood using for the first time single-cell Mass Cytometry (CyTOF). We were able to measure the expression of 31 surface markers within a single red blood cell (RBC). We demonstrate the validity of CyTOF for RBC phenotyping by confirming the progressive reduction of transferrin receptor 1 (CD71) during reticulocyte maturation to mature RBC. We highlight the high-dimensional nature of mass cytometry data by correlating the expression of multiple proteins on individual RBCs. We further describe a more drastic reduction pattern for a component of the alpha4/beta1 integrin CD49d at the very early steps of reticulocyte maturation in bone marrow and directly linked with the mitochondria remnants clearance pattern. The enhanced and accurate RBC phenotyping potential of CyTOF described herein could be beneficial to decipher RBC preferences, as well as still not well understood receptor-ligand interaction of some hemotropic parasites such as the malaria causing agent *Plasmodium vivax*.

1. Introduction

Red blood cells (RBCs) are among the most uniform cell populations in the human body. The youngest RBCs in peripheral circulation, the reticulocytes, represent the endpoint of erythropoiesis in the bone marrow [1, 2]. To complete erythropoiesis, central macrophages in erythroblastic islands play instrumental roles providing iron, secreting cytokines, erythropoietin and transcription factors [3] and, very importantly, they perform phagocytosis of the protruding nuclei from the surrounding orthochromatic erythroblasts [4]. Junction maintenance of erythroblastic islands is via αv integrins, VCAM-1 and EMP in macrophages interacting with ICAM-4, VLA-4 and EMP in erythroblasts [5, 6]. A complex number of molecules serves as intermediates in this process (CD151, CD81, CD82, CD44, CD98, CD147, CD163, CD169 and Siglec-1) [7–12]. Once enucleated, these cells become reticulocytes [13], which are released to the peripheral circulation to develop within 40 h [14] to fully mature RBCs. In conjunction with this developmental step, the reticulocyte membrane switches from an endocytic state [15] to a preponderant exocytic status as RBCs mature in peripheral blood circulation. This leads in the reticulocyte-to-mature-RBC transition to a reduction of the surface membrane by expelling excess surface proteins [14]. Moreover, remnants of internal organelles such as ribosomes, endoplasmic reticulum (ER) and Golgi apparatus are also cleared, although their underlying mechanisms are not completely determined [16]. A macroautophagy pathway called mitophagy seems to be responsible for mitochondria degradation and clearance; yet, how early in reticulocyte maturation this is established is also not well understood.

Historically, the larger more amorphous reticulocytes [17] in peripheral circulation are also defined microscopically by their typical reticular network visible with supra-vital stains such as New Methylene Blue (NMB) and sub-classified in 4 Heilmeyer stages (I–IV) [18]. A classification for reticulocytes into R1, which would correspond to the more immature fraction of reticulocytes observed after enucleation in the bone marrow, and R2, located in peripheral blood, has been initially proposed [19]. However, nowadays RNA loss and above all the surface expression of CD71are the most common markers used to define the

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maturation from reticulocytes to fully mature RBCs [14]. Immature reticulocytes express higher levels of CD71 whereas during maturation, CD71 levels are reduced to barely measurable. Although composition, abundance, and changes in reticulocyte membrane proteins have been already studied during maturation [20–22], current single-cell studies based on flow cytometry have been limited to the number of spectrally resolvable fluorochromes [23].

Mass Cytometry (CyTOF) is the adaptation of Inductively Coupled Plasma Mass Spectrometry to single-cell analysis [14] and offers the ability to measure up to 40 parameters within a single cell [24–26]. This study is the first report to apply the powerful CyTOF phenotyping technology to characterize RBCs. Here, we bring a better understanding for the complexity of reticulocyte maturation from bone marrow to peripheral blood and suggest very immature reticulocyte surface molecules such as CD49d to be worth testing upon their role upon *Plasmodium vivax* (*P. vivax*) invasion mechanism.

2. Materials and method

2.1. Reticulocytes and mature RBCs

Four Duffy$^+$ (samples 1–4) and four Duffy$^-$ (samples 5–8) fresh reticulocyte and mature RBCs preparations (in total 8 peripheral blood samples) were obtained from Buffy coat (BC) packs (The Interstate Blood Bank INC. Memphis, USA). BC blood was centrifuged at 2500*g* for 5 min, washed with McCoy 5A incomplete Medium (MIM) (Sigma M4892-10L) and filtered for leuko-depletion on a NEO Purecell Leukocyte Reduction filter (Haemonetics). To separate reticulocytes from mature RBCs, we performed CD71 immuno-magnetic purification following the manufacturer's specifications (Miltenyi Biotech). CD71$^+$ and CD71$^-$ RBCs were separated on an autoMACS Pro Separator (Miltenyi Biotech), washed and re-suspended in MIM. NMB thin blood smears were performed after a 1:1 dilution and 15 min incubation at 37 °C to check for purity (generally above 95%). Reticulocytes and mature RBCs were kept at 4 °C in MIM until used, always before 2 days post isolation. One bone marrow reticulocyte preparation was obtained from unprocessed whole aspirates (ALLCELLS) following the same protocol as for peripheral blood BC packs, by which nucleated erythroblasts were retained in the Leukocyte Reduction filter. Residual WBC contamination (CD45+ cells) was observed in our peripheral blood samples and slightly higher for bone marrow samples.

2.2. Mass cytometry

Commercially available metal-isotope-tagged antibodies against RBC surface markers were purchased from Fluidigm. When non-available, non-conjugated purified antibodies lacking carrier proteins were purchased from other companies (Table 1) and these antibodies were custom-conjugated by Fluidigm with heavy metal-preloaded maleimide-coupled MAXPAR chelating polymers (Table 1). A validation step was performed by Fluidigm to assure their viability (Supplemental Fig. 1). CD233 (Band 3) validation was not acceptable and therefore not taken into account for the ulterior analysis. CD234 (DARC) showed lower intensity signal than expected, yet it was acceptable for our purposes of distinguishing the different Duffy blood groups in our samples. In order to determine the differences in surface markers within maturation in RBCs we optimized the company's recommended protocol into a 3 days staining protocol for every sample. On day 1, 1×10^7 mature RBCs or reticulocytes were re-suspended in PBS and Cell-IDTM Cisplatin (Fluidigm) to a final concentration of 5 μM. The mixture was incubated at RT for 5 min followed by a quench staining with MaxPar Cell Staining Buffer (MCSB) (Fluidigm) using 5× the volume of the cell suspension. For antibody staining, the RBC pellet was re-suspended in 50 μL of MCSB. Then 50 μL of metal isotopes labeled antibody cocktail prepared in MCSB, following the company's specifications, was added giving a total volume of 100 μL. Samples were vortexed, incubated for 30 min at RT and washed twice by adding 2 mL of MCSB, centrifuged and the supernatant discarded. We re-suspended our cells in 1 mL of a 0.05% glutaraldehyde solution (Sigma) and set at 4 °C for overnight shipment to The Human Immune Monitoring Center (HIMC), Stanford University, Palo Alto, California. Upon arrival on day 2, cells were centrifuged at 800*g* and washed twice with 1 mL of MCSB. The pellet was then incubated in 100 μL of MaxPar Fix/perm Buffer at 4 °C for 30 min and then re-suspended in 1 mL Cell Intercalation Solution containing Cell-IDTM Intercalator-Ir (Fluidigm) to a final concentration of 125 nM, mixed and incubated overnight at RT. On day 3, cells were washed 2 times with 2 mL of MCSB and 2 more times with MilliQ water (Millipore). Prior to CyTOF run, pellet was adjusted to 2.5–5 × 10^5/mL. Data was acquired on a CyTOF 2 (Fluidigm) at HIMC. Data obtained by Mass Cytometry was converted to a FCS file and analyzed by FlowJo software (Treestar Inc.). We first gated out EQ-beads used for internal compensation. We set 10^1 as a threshold for negativity/background using BIP (a *Plasmodium* spp. specific C-terminal peptide epitope involved in endoplasmic reticulum retrograde trafficking) that should not be expressed on human RBCs. Using this 10^1 threshold, we gated out CD45+ cells to skip analyzing any remnants of white blood cells post leuko-depletion. We were able to fully analyze 43.465 to 234.004 events per sample. For every RBC marker, we determined the positive and the negative population based on the 10^1 set threshold for negativity. Moreover, for every RBC marker, either positive or negatively expressed, we determined the expression of the whole set of markers to determine their correlation (i.e. CD41$^+$-CD49d$^+$/CD41$^+$-CD49d$^-$/CD41$^-$-CD49d$^+$/CD41$^-$-CD49d$^-$).

2.3. Computational methods and statistical analysis

2.3.1. Generation of a marker intensity matrix

In order to represent and analyze the data obtained by mass cytometry regarding the intensity of 31 RBC markers for each single cell in sample s ($s \leq 12$, $s \in$ N1–N4, R1–R8) we generated a matrix M^s_{ij}, where i indicates each single cell in specific samples ($i \leq$ the total number of cells in sample s); and j indicates the specific RBC marker ($1 \leq j \leq 31$).

2.3.2. RBC markers correlation between different RBCs type

For each sample s, we calculated the number of cells N_j with specific marker j. It can be represented as:

$$N_i = \sum_i 1(M_{i,j} > 10)$$

here, 1 represents the indicator function. Thus, we have a vector (N^s_1, N^s_2, N^s_3,… N^s_{31}) to represent each sample s. The Pearson correlation can be calculated for each pair of samples.

2.3.3. CD71 marker threshold selection

For mass cytometry analysis, reticulocytes from R1–R4 samples were classified in 2 groups based upon CD71 intensity for peripheral blood (< 400 & > 400). We choose this cut-off based on the estimated background signal obtained in fully mature RBCs. The quantile distribution of the CD71 intensity in mature RBCS is 4.69 for 0%, 23.18 for 25%, 45.47 for 50%, 99.32 for 75% and 381.33 for 100%. Since the biggest CD71 in N cell was ~382, we choose 400. With the threshold 400, over 99.9999% cells show non-significant CD71 levels.

2.3.4. Visualization of multiple single-cell data sets

We used the cloud-based platform Cytobank (www.cytobank.org), for analysis and visualization of multiple single-cell data sets simultaneously. We used SPADE (spanning-tree progression analysis for density-normalized events) to cluster over 100,000 sets of single-erythroid data into phenotypically similar cells of hierarchical trees.