Regulation of complement activation on red blood cells

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Chapter 3

Complement regulator CD46 expression is lost gradually during erythropoiesis and enucleation

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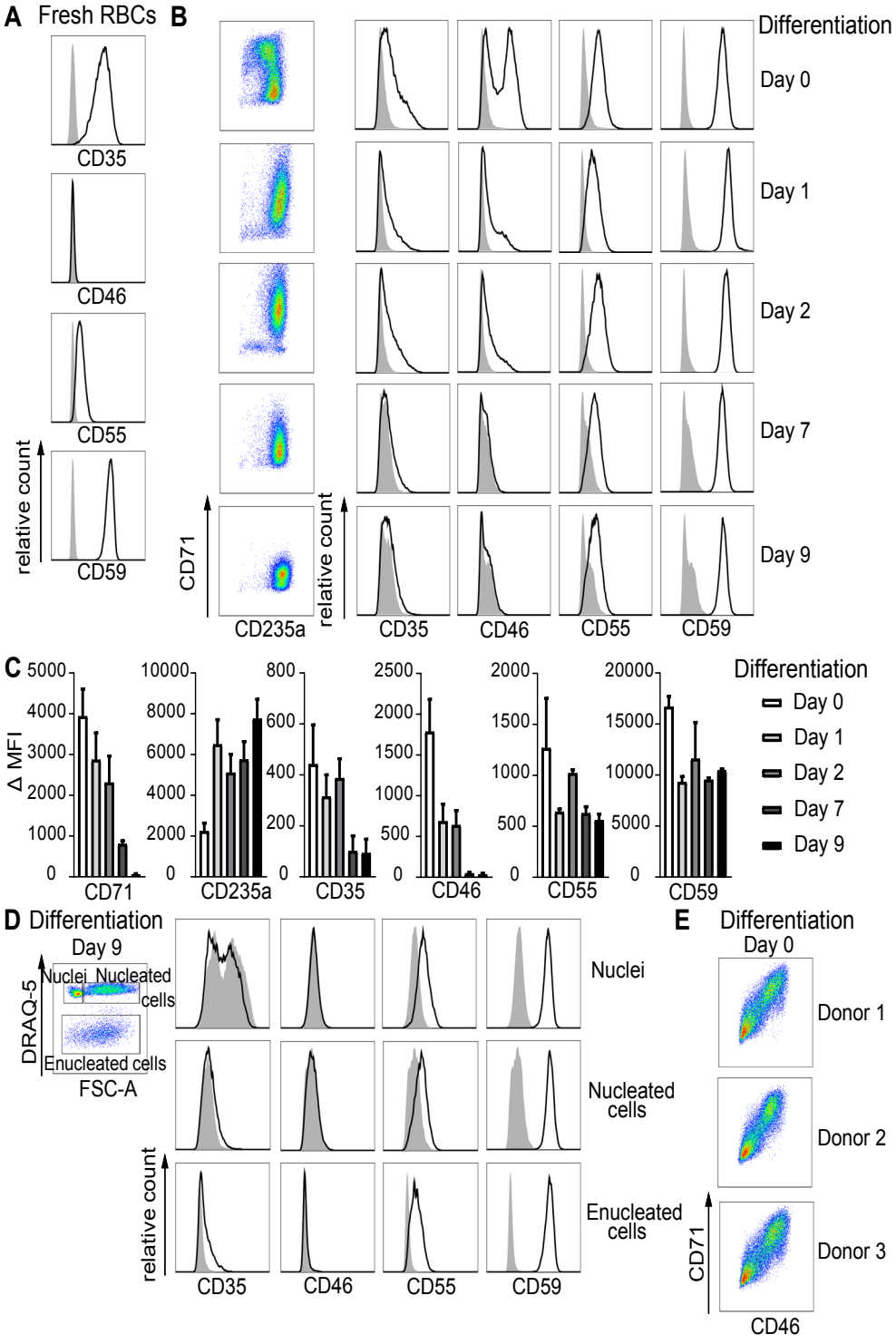
TO THE EDITOR

Complement regulator CD46 expression is lost gradually during erythropoiesis. Erythropoiesis occurs in the bone marrow and starts with a hematopoietic stem cell that differentiates via pro-erythroblast to normoblast that expel their nuclei to form a reticulocyte that exits the bone marrow and finally matures to red blood cells (RBCs) in the blood. This differentiation process is accompanied by cell size decrease, chromatin condensation, hemoglobin accumulation and sorting of specific membrane proteins during enucleation and upon final maturation in the blood[1-3]. About 2 million reticulocytes are produced per second and during anemia this may be increased up to 20-fold[1]. Circulating RBCs are continuously in contact with complement components dissolved in blood plasma[4]. The complement system is an essential component of our innate immune system that is involved in the clearance of pathogens, dying cells and immune complexes. Fluid phase and membrane bound complement regulators offer protection against ongoing complement activation and inhibit complement-mediated RBC destruction. On the membrane of RBCs, complement regulators CD35, CD55 and CD59 are expressed. In contrast, the membrane bound complement regulator CD46 is expressed on nucleated cells, but is absent on mature RBCs[4-6]. We hypothesized that CD46 is lost upon enucleation by preferential sorting to the membrane of the expelled nucleus. In the present study, we investigated the expression dynamics of CD46 during erythropoiesis.

To address this question, hematopoietic stem and progenitor cells (HSPC) within peripheral blood mononuclear cells (PBMCs) of three healthy donors were differentiated to proerythroblasts and subsequently, matured to reticulocytes within 9 days as described previously[7]. As described by others, healthy donor RBCs expressed complement regulators CD35, CD55 and CD59, but lacked the expression of CD46 (Fig. 1A). Erythroblast differentiation to enucleated reticulocytes is accompanied by upregulation of CD235 (glycophorin A) and a decreasing expression of CD71 (holotransferrin receptor) marking the different stages of erythropoiesis (Fig. 1 B) and in line with previous publications[8,9]. Complement regulators CD55 and CD59 were present at the erythroblast stage and expression remained stable during differentiation (Fig. 1B and Fig. 1C). The expression of CD46 decreased over time and was lost between day 2 and day 7 (Fig. 1B and 1C), which is between the early erythroblastic and orthochromatic normoblastic stage. To determine whether CD46 was specifically lost during the process of enucleation, a DNA stain (DRAQ5) was used to distinguish between nuclei, nucleated and enucleated cells. However, no expression of CD46 was observed on either of these populations (Fig. 1D), which was in line with the early loss of CD46 observed in Fig. 1B. In contrast, complement regulators CD55 and CD59 were expressed on nuclei, nucleated and enucleated cells (Fig. 1D). To confirm whether the dynamics of


A Fresh RBCs
CD35
CD46
CD55
CD59
relative count
B
Differentiation
Day 0
Day 1
Day 2
Day 7
Day 9
CD71
CD235a
relative count
CD35
CD46
CD55
CD59
C
Δ MFI
CD71
CD235a
CD35
CD46
CD55
CD59
Differentiation
Day 0
Day 1
Day 2
Day 7
Day 9
D Differentiation
Day 9
Nuclei
Nucleated cells
Enucleated cells
DRAQ-5
FSC-A
Nuclei
Nucleated cells
Enucleated cells
relative count
CD35
CD46
CD55
CD59
E Differentiation
Day 0
Donor 1
Donor 2
Donor 3
CD71
CD46

CD46 regulation during *in vitro* is reflected *in vivo*, erythropoiesis within bone marrow of a healthy donor was investigated. Fig. 2A shows that CD46 is expressed at the erythroblast stage, but gradually lost during differentiation, which is in agreement with the *in vitro* culture data. The CD71lowCD235+ late normoblast & reticulocytes gate represents a mix of nucleated DRAQ5+ normoblasts and DRAQ5-immature enucleated reticulocytes[10]. *In vivo*, we observed that CD46 is lowly expressed on nucleated late normoblasts and is almost not expressed on enucleated reticulocytes, suggesting that the reminiscent CD46 is lost during enucleation (Fig. 2B). So, both *in vitro* and *in vivo* CD46 is predominantly lost during early differentiation and the remaining CD46 is finally routed to the pyrenocytes during enucleation. The lack of CD46 on mature RBCs might have an evolutionary benefit. Different pathogens such as viruses (measles, human herpes virus 6 and different serotypes of adenovirus) and bacteria (Streptococcus pyogenes and Neisseria species) use CD46 as an entry receptor[11]. It has been proposed that the lack of CD46 on mature RBCs prevents dissemination of pathogens as the enucleated reticulocytes are incapable to support virus replication[12,13].

Remarkably, the expression of CD46 resembles a similar decreasing expression pattern as CD71 during *in vitro* (Fig. 1E) and *in vivo* (Fig. 2C) erythropoiesis. This indicates that the expression of both CD46 and CD71 is decreasing at the same differentiation stage of erythropoiesis and thus CD46 may be used as a surrogate erythroid differentiation progression marker replacing CD71, thereby increasing the panel of markers that can be used to monitor progression of erythropoiesis. It has been described that during erythropoiesis and reticulocyte maturation into mature RBCs, CD71 is respectively preferentially routed to the pyrenocytes and released via exosomes during further reticulocyte maturation. Indeed, other proteins such as acetylcholinesterase, nucleoside transporters, α4β1-integrin, aquaporin-1, and GPI-anchored proteins including complement regulators CD55 and CD59 can also be found in reticulocyte exosomes[14-17]. In contrast to CD71, regulators CD55 and

← **Figure 1: Complement regulator CD46 is lost before enucleation during in vitro differentiation.** **(A)** Representative FACS plots are shown of complement regulators CD35, CD46, CD55 and CD59 on fresh RBCs from three healthy donors. **(B-E)** PBMCs from the same three healthy donors were isolated and expanded/differentiated as previously described[7]. **(B)** Representative FACS plots are shown for CD71 and CD235a that illustrate the various differentiation stages and their expression for complement regulators CD35, CD46, CD55 and CD59 are shown. **(C)** Bar graph of CD71, CD235a, CD35, CD46, CD55 and CD59 for the different days are shown. Data is presented as mean ± standard deviation, n=3 donors, MFI: mean fluorescence intensity, ΔMFI = MFI – background. **(D)** At day 9, a DNA stain, DRAQ 5, was used to discriminate between nuclei, nucleated cells and enucleated cells and expression of complement regulators CD35, CD46, CD55 and CD59 are shown. **(E)** FACS plots of the correlation between CD71 and CD46 for the three healthy donors are shown.

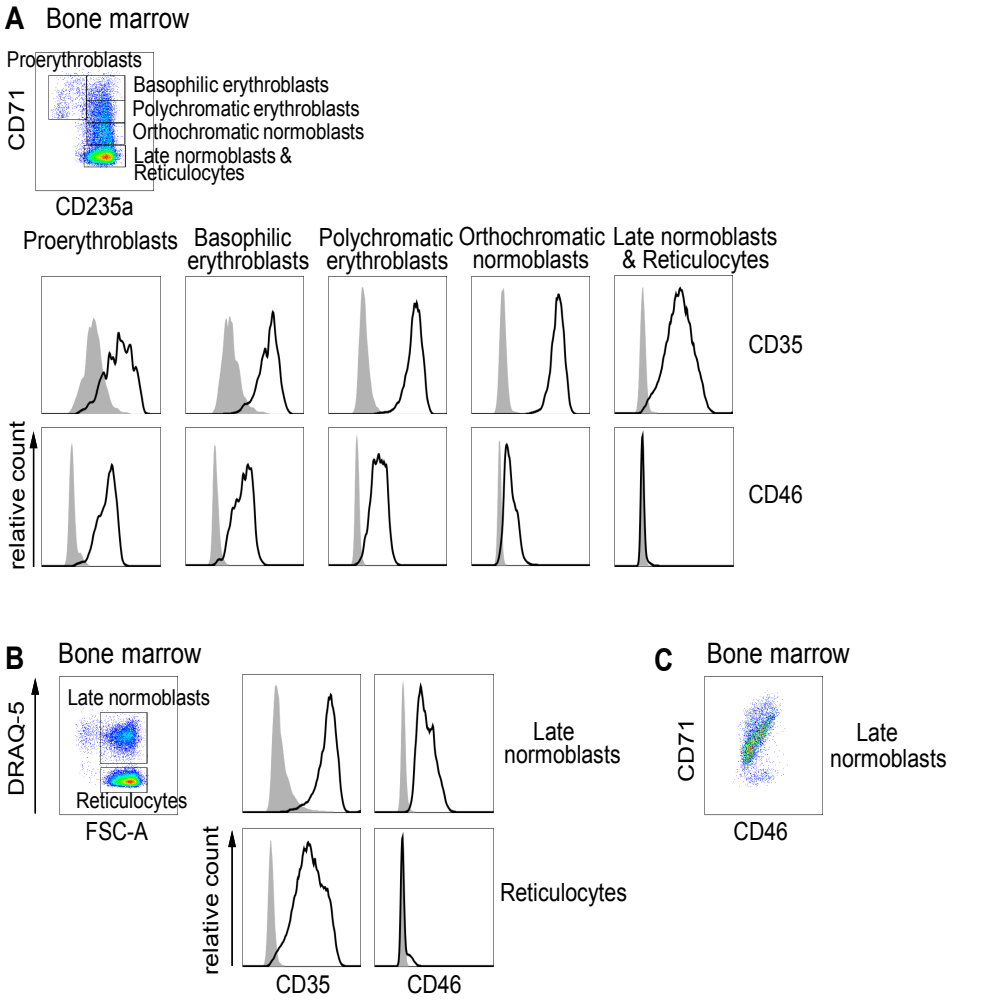


Figure 2: Complement regulator CD46 is lost during erythropoiesis, while CD35 is expressed in all in vivo developmental stages. (A) FACS plots displaying a CD71/CD235 staining of healthy donor bone marrow is shown and the various developmental stages are indicated. Histograms show the expression of complement regulators CD35 and CD46. **(B)** The DNA stain DRAQ 5 was used to discriminate between late normoblasts and reticulocytes of the bone marrow sample and the expression of complement regulators CD35 and CD46 are shown. Nuclei are not present as these are phagocytosed by central macrophages in vivo. **(C)** FACS plot of the correlation between CD71 and CD46 in the bone marrow sample is shown.

CD59 are still expressed on mature RBCs. This might be because CD55 and CD59 are anchored to the cell membrane, in analogy to rhesus proteins, and CD46 and CD71 are not[3]. However, whether CD46 is tethered or associated with the erythroid spectrin cytoskeleton remains unknown.

Moreover, we observed expression of CD35 on day 0, 1 and 2 of differentiation, while low expression of CD35 was observed on day 7 and 9 (Fig. 1B and 1C). However, as shown in Fig. 1A using the same CD35 antibody, CD35 is expressed on mature RBCs of these healthy persons. So low CD35 expression at the end of erythroid differentiation was unexpected. In contrast, we observed that CD35 was expressed on all differential stages of the bone marrow sample (Fig. 2A) and also was present on both nucleated late normoblasts and enucleated reticulocytes (Fig. 2B). Apparently, expression of CD35 differs between the erythroid maturation stages within the bone marrow sample and *in vitro* differentiated cells, indicating that the cultured cells not completely resemble the *in vivo* situation.

CD35 acts as co-factor for factor I in the inactivation of complement deposition and is important for the clearance of complement opsonized immune complexes, called immune adherence[5]. Therefore, it might be that *in vitro* differentiated cells are more vulnerable for complement attack and have less immune adherence capacity. However, persons with the knops 'null' phenotype that display extremely low expression of CD35 have no health problems[18-20]. So, it has to be further investigated whether decreased expression of CD35 on *in vitro* differentiated cells has any functional consequences.

In conclusion, our results showed that CD46 follows a similar decreasing expression pattern as CD71 during erythropoiesis and this occurred mainly before enucleation. The complement regulators CD55 and CD59 are expressed during all development stages of erythropoiesis. Remarkably, CD35 was already expressed early in erythropoiesis showing that proerythroblasts uniquely express both CD35 and CD46 complement regulators.

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