Cellular mechanisms involved in seronegative RBC hemolysis

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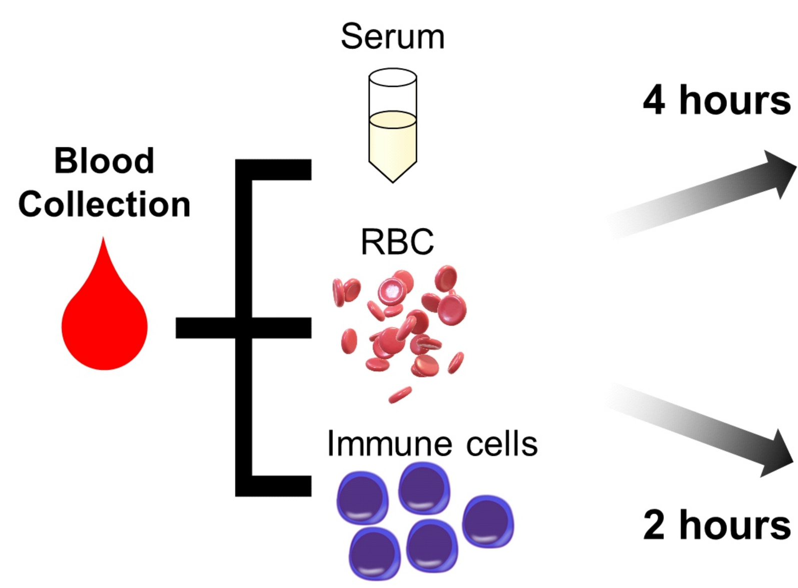
Abstract: Hemolysis is a potentially life-threatening complication involving the rupture of red blood cells (RBC) associated with several diseases, including AIHA, sickle cell disease (SCD), cancer and ITP among others. Causes of hemolysis can be intrinsic or extrinsic, the latter being of non-immune or immune nature. With the current dogma of immune hemolysis being mediated by humoral responses, lab tests rely on the detection of antibodies bound to RBC or present in the plasma. However, in some cases, serological investigations results are negative, without alternative explanations for hemolysis occurrence. Therefore, we decided to investigate cellular mechanisms possibly causing hemolysis when there is no evidence of antibody involvement. To evaluate cell cytotoxicity against RBCs, release of Cr^{51} was selected as the most sensitive method. Effector cells of the immune system (NK cells, neutrophils, monocytes, M1/M2 macrophages) were evaluated as mediators of RBC lysis or phagocytosis. We observed that NK cells are the most efficient in killing anti-D coated RBC when compared to PBMC or monocytes. Neutrophils are also capable to lyse RBC when primed with relevant cytokines. These results suggest that cellular mechanisms besides extravascular antibody-mediated phagocytosis may play a role in hemolysis. Our preliminary results in some seronegative hemolysis patients show evidence of cell-mediated RBC lysis. Additionally, M2 macrophages were able to mediate phagocytosis of RBC expressing phosphatidylserine when opsonized with SCD patients' serum. However, inclusion of more patients is required to further document the cell-mediated cytotoxicity in seronegative hemolysis and to elucidate this phenomenon of M2 macrophage-mediated phagocytosis in SCD.

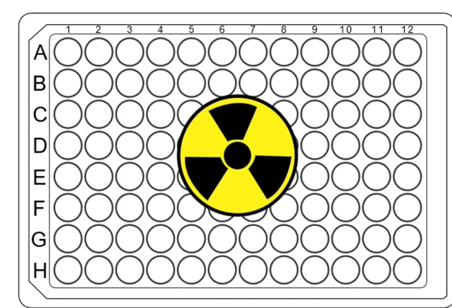
Methods:

Comparison of RBC lysis detection methods

- QuantiChrom™ Hemoglobin Assay Kit — BioAssay Systems (Solutions for Research and Analysis)
- Cytotoxicity Detection Kit PLUS (LDH) — Roche
- Chromium-51 Radionuclide — PerkinElmer (For the Better)

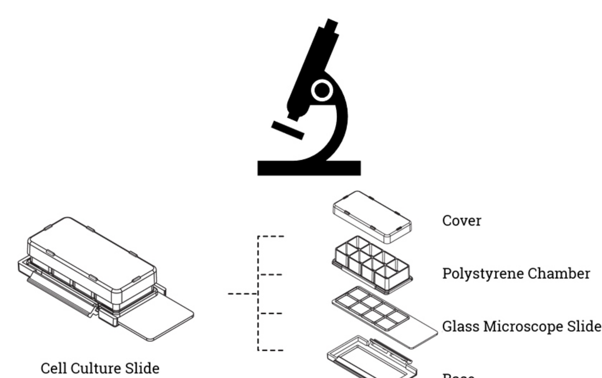


4 hours



2 hours

Phagocytosis assay



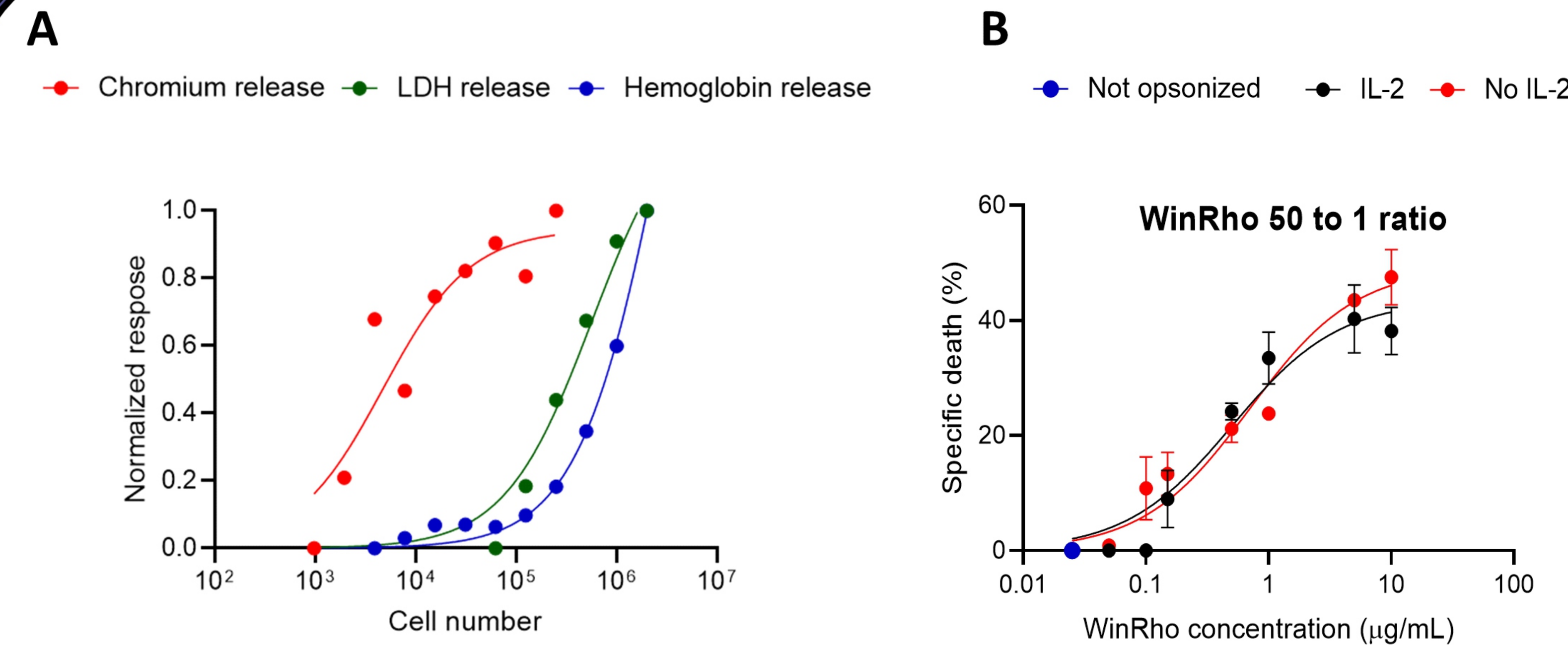


Figure 1. Cell lysis method selection and standardization. (**A**) Hemoglobin release, LDH release and Cr^{51} release were evaluated to assess their feasibility to detect RBC death in the most sensitive manner. Different cell numbers were lysed by adding HCl or 1% Triton X100 and cell death evaluated 4 hours later. Death was detectable by all methods, but Cr^{51} release resulted in the best method to detect significant levels of death with the lower cell number. (**B**) As positive control of immune cell mediated RBC lysis in our system, we decided to opsonize R2R2 RBC with WinRho (anti-D antibody). We coated RBC with different WinRho concentrations, co-incubated with PBMC (50 to 1 effector:target ratio) and performed a Cr^{51} release assay to measure cell cytotoxicity (ADCC). We selected opsonization with 5µg/mL as the lowest antibody concentration that induced a maximum amount of death.

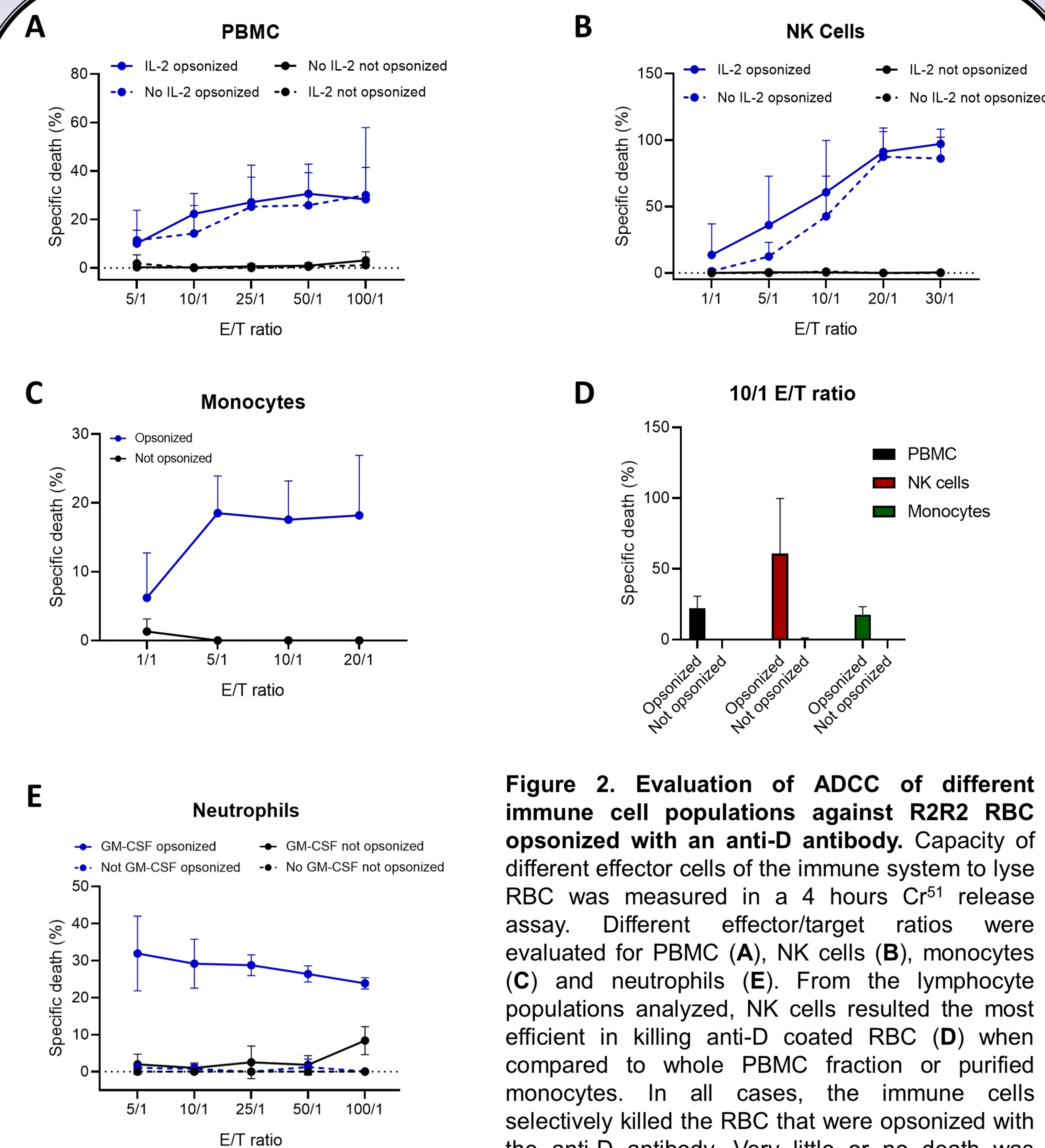


Figure 2. Evaluation of ADCC of different immune cell populations against R2R2 RBC opsonized with an anti-D antibody. Capacity of different effector cells of the immune system to lyse RBC was measured in a 4 hours Cr^{51} release assay. Different effector/target ratios were evaluated for PBMC (**A**), NK cells (**B**), monocytes (**C**) and neutrophils (**E**). From the lymphocyte populations analyzed, NK cells resulted the most efficient in killing anti-D coated RBC (**D**) when compared to whole PBMC fraction or purified monocytes. In all cases, the immune cells selectively killed the RBC that were opsonized with the anti-D antibody. Very little or no death was observed in non-opsonized RBC, making our model suitable to be used as a positive control.

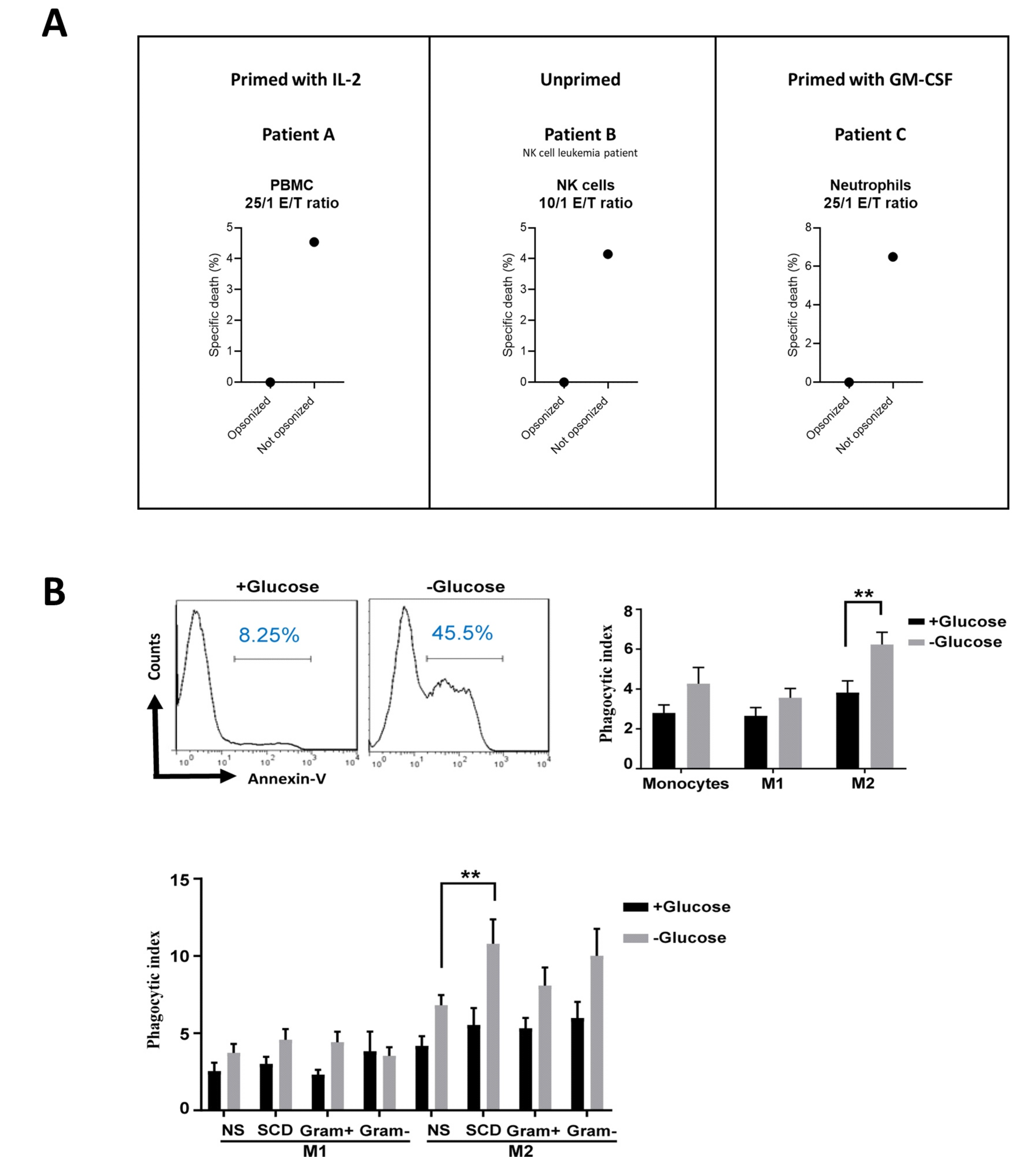


Figure 3. Cell-mediated RBC lysis or phagocytosis in patients with seronegative hemolysis. Blood was collected from seronegative hemolysis patients after informed consent and immediately transported to the lab for cell isolation. Different immune cell populations, RBC and serum were isolated from each patient and samples were prepared for Cr^{51} release or phagocytosis assays. For the Cr^{51} release assay, RBC were opsonized or not with patient's own serum and incubated with the respective immune effectors. RBC specific killing was evaluated after 4 hours of incubation. (**A**) Representative examples of patients that showed evidence of direct RBC killing by at least one of the immune cells evaluated.

For the phagocytosis assay, monocytes were derived to M1 or M2 macrophages with the addition of cytokines. SCD patients are known to express Phosphatidylserine (PS) on the outer membrane layer of the RBC. To mimic such scenario, we deprived RBC from Glucose, observing an increase on PS expression by Annexin-V staining (**B**). We incubated those RBC with the cultured macrophages and interestingly, we observed a preferential phagocytosis mediated by M2 macrophages. This finding was also observed when autologous RBC from SCD patients were opsonized with the patient's serum. Similar results were obtained for sepsis patients, which also have an increased expression of PS on the RBC membrane.

Conclusions:

1. Anti-D opsonization of R2R2 RBC is a suitable model to evaluate the ADCC capacity of immune cells from different lineages.
2. Cellular mechanisms seem to be involved in the RBC lysis of patients with seronegative hemolysis, at least in our *in vitro* scenario.
3. Phagocytosis of RBC from patients with SCD is preferentially mediated by M2-derived macrophages

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