ANTIBODIES ABLE TO INDUCE ANTIBODY MEDIATED-IMMUNE SUPPRESSION PROMOTE *IN VITRO* ANTIGEN-LOSS BY MACROPHAGES

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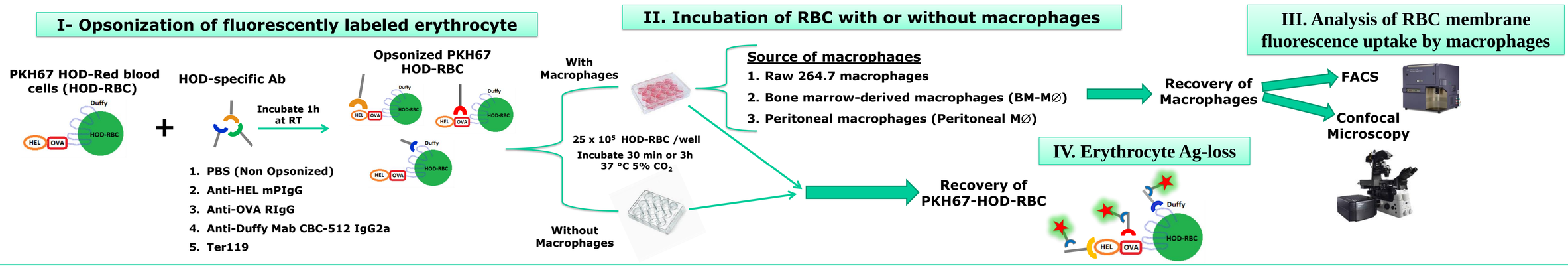
INTRODUCTION

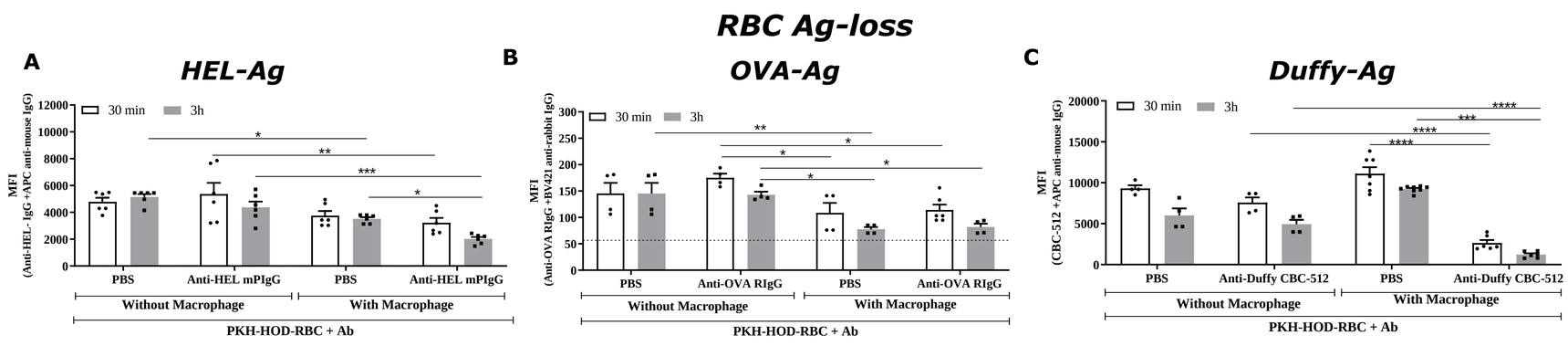
Hemolytic disease of the fetus and newborn (HDFN) is an alloimmune condition that occurs when maternal antibodies specific to fetal red blood cell (RBC) antigens cross the placenta and cause fetal RBC destruction or fetal bone marrow suppression of RBC progenitors [1, 2]. Polyclonal anti-RhD antibodies (anti-D) is the only therapy available to prevent HDFN [3, 4] and this mechanism has been referred to as antibody-mediated immune suppression (AMIS) [5]. Unfortunately, anti-D is a human blood product vulnerable to shortages, and the mechanism of AMIS is unknown. Our laboratory has previously explored the mechanism of AMIS using a mouse system expressing the synthetic hen egg lysozyme-ovalbumin-Duffy (HOD) antigen on RBCs (HOD-RBC) (6,7,8). Other and us have identified that AMIS is closely linked with the ability of antibodies to mediate antigen loss (9,10). Unfortunately, the RhD on RBC is not immunogenic in standard laboratory mice [11], for this reason several alternative mouse models have been developed. However, there is no available mouse model to study AMIS ability of RhD-specific Abs. Then, *in vitro* Ag-loss evaluation can be an alternative to evaluate the AMIS-inducing potentialities of new recombinant antibodies and find a monoclonal antibody to replace anti-D. The aim of the present work was to determine the ability of AMIS-inducing antibody to promote *in vitro* Ag-loss.

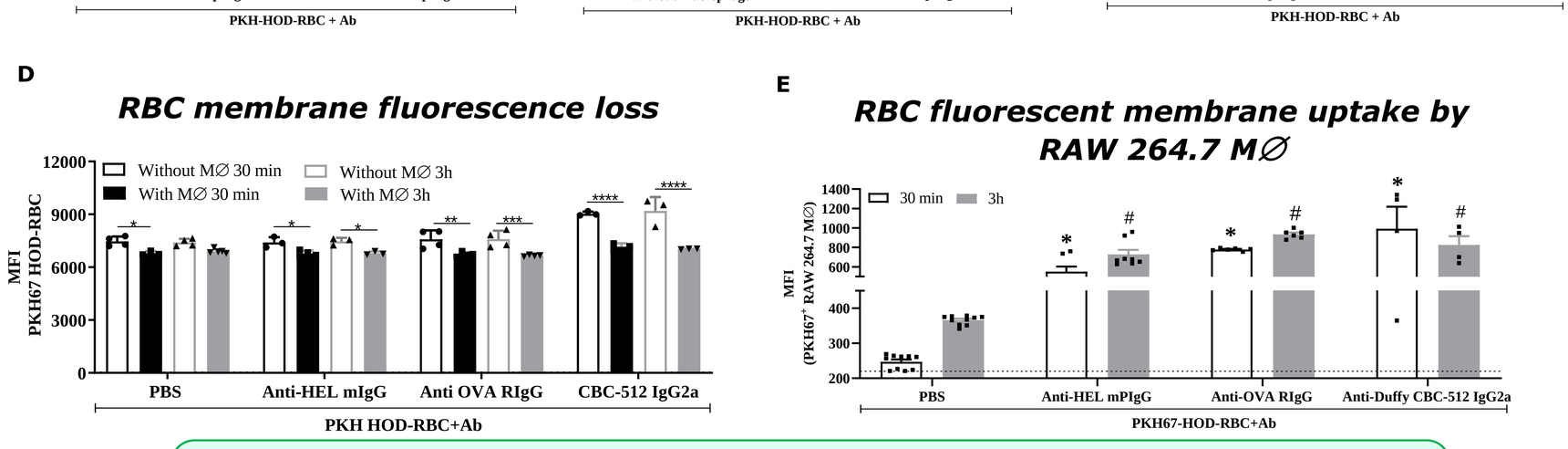
MATERIALS AND METHODS

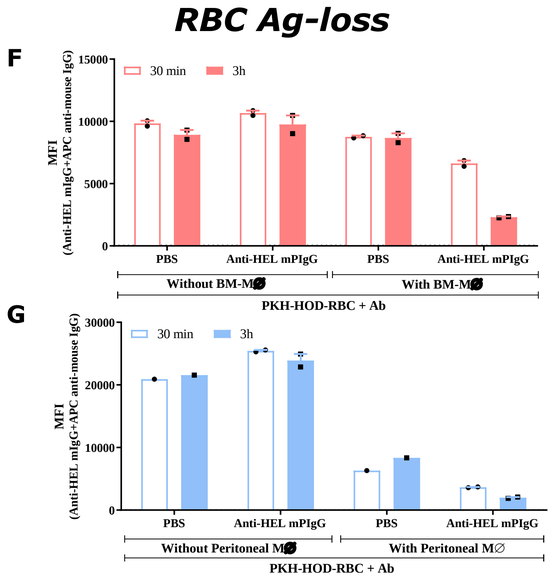


RESULTS

AMIS-inducing antibodies promote *in vitro* erythrocyte Ag-loss by different types of macrophages



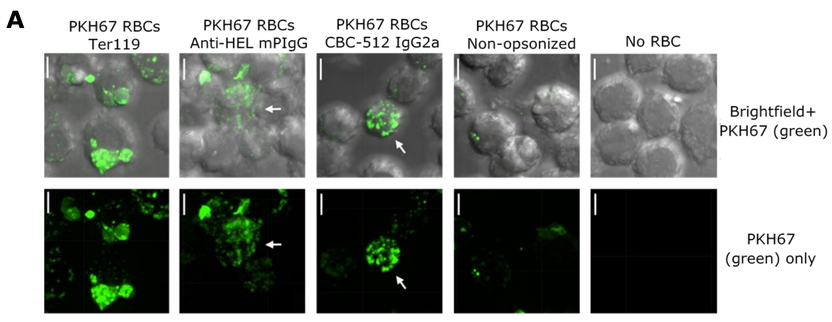


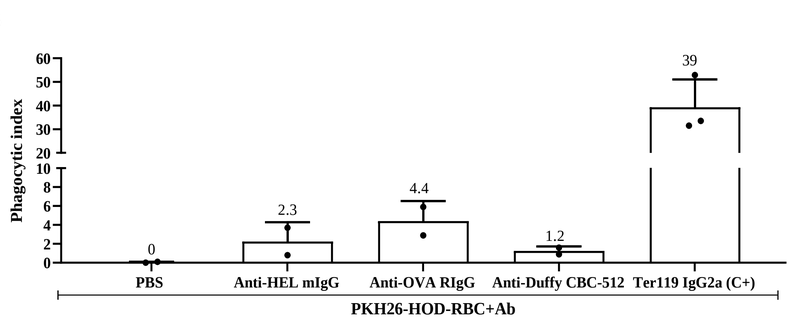


HOD-specific antibodies able to induce AMIS provoked *in vitro* erythrocyte Ag-loss in both a macrophage- and antibody-dependent manner and reached maximal levels within 3 hours

Figure 1. AMIS-antibody induces *in vitro* erythrocyte Ag-loss with fluorescent membrane transfer to macrophages. RBC from HOD mice were stained with the membrane dye PKH67. PKH67-HOD-RBC were opsonized with the AMIS-inducing antibodies (anti-HOD antibodies; anti-HEL mPIgG, Anti-OVA RIgG, and anti-Duffy mAb CBC-512 IgG2a) or non-opsonized and then co-incubated with macrophages (MØ) for 30 min and 3h in RPMI 10% FBS (37°C, 5% CO2). Fluorescent HOD-RBC (opsonized vs not) were also incubated without (MØ) in the same condition. After a period of incubation HOD-RBC were recovery and macrophages were washed, and remaining RBCs lysed by hypotonic lysis. **A**) HEL-Ag **B**) OVA-Ag and **C**) Duffy-Ag detection on surface of erythrocytes recovered were performed incubating with anti-HEL mPIgG, anti-OVA RIgG or CBC-512 plus APC labeled anti-mouse IgG or anti-rabbit IgG. RAW macrophages were washed **E)** their PKH67 MFI were evaluated. Both samples erythrocytes and macrophages were analyzed by flow cytometry. Similar experiment was performed using bone morrow-derived macrophages (BM-MØ) or peritoneal macrophages (Peritoneal MØ) as a phagocytic cells. HEL-Ag on surface of erythrocytes non-opsonized or opsonized recovered from incubation with **F)** BM-MØ and **G**) PMØ was evaluated as described in A. Macrophages were washed and **H)** their PKH67 MFI were evaluated. Statistical analyses (A, B and C): Two-way ANOVA and Sidak's multiple comparisons test; (E) Two-way ANOVA and Dunett's multiple comparisons test (*) and (#) indicate significant difference (p<0.05) between non-opsonized vs opsonized samples at 30 min and 3h respectively.

HOD-specific antibodies with AMIS ability induce erythrocyte membrane transference to macrophages without phagocytosis





AMIS-inducing antibodies did not lead to detectable macrophage phagocytosis of HOD-RBCs despite induction of erythrocyte membrane uptake

Figure 2. Anti-HOD antibodies mediate HOD RBC membrane transfer to RAW 264.7 macrophages but they did not induce phagocytosis. RAW 264. 7 macrophages were seeded on coverslips two days prior. HOD-RBC were collected and labeled with a membrane dye PKH67. Labeled HOD-RBCs were opsonized with Anti-HEL mPIgG, Anti-OVA RIgG and Anti-Duffy CBC-512 IgG2a or kept non-opsonized before being co-incubated with macrophages in RPMI + 10% fetal bovine serum (37°C, 5% CO2) for 30 min (Phagocytosis) or 3h (Confocal microscopy). The anti-Ly76 antibody TER-119 was used as a positive control. Later, excess RBCs were removed and remaining RBCs were lysed by hypotonic lysis. Macrophages were washed, fixed with formaldehyde, and **A)** imaged on a Quorum multi-modal imaging system (spinning disc confocal) under 63x objective oil immersion. Scale bar = 5 µm. Arrows point towards macrophages with punctate staining possibly due to trogocytosis of HOD RBC membrane. **B)** The phagocytic index was calculated as the number of erythrocytes engulfed per 100 macrophages.

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CONCLUSIONS

1. AMIS-inducing antibodies can mediate macrophage-driven antigen loss from RBC *in vitro*.
2. Erythrocyte Ag-loss may occur by a trogocytosis-type mechanism where RBC membrane and antigen is transferred to the macrophage without phagocytosis.