Published in final edited form as:

Transfusion. 2019 January ; 59(1): 371–384. doi:10.1111/trf.14939.

Antibody-mediated immunosuppression can result from RBC antigen loss independent of Fc γ receptors in mice

Amanda Mener, Seema R. Patel, Connie M. Arthur, Sean R. Stowell

Center for Transfusion Medicine and Cellular Therapies, Department of Laboratory Medicine and Pathology, Emory University School of Medicine, Atlanta, Georgia.

Abstract

BACKGROUND: Anti-RhD administration can prevent *de novo* anti-RhD formation following RhD+ red blood cell (RBC) exposure, termed *antibody-mediated immunosuppression* (AMIS). Recent studies suggest that AMIS may occur through target antigen alterations, known as antigen modulation. However, studies suggest that AMIS may occur independent of antigen modulation. In particular, AMIS to RBCs that transgenically express the fusion hen egg lysozyme-ovalbumin-Duffy (HOD) antigen have been shown to occur independent of activating Fc γ receptors (Fc γ Rs) thought to be required for antigen modulation. Therefore, we sought to determine the mechanism behind AMIS following HOD RBC exposure.

STUDY DESIGN AND METHODS: Following transfer of HOD RBCs into wild-type or Fc γ R-chain knockout recipients in the presence or absence of monoclonal anti-hen egg lysozyme (HEL) antibody, individually or in combination, HOD antigen levels and anti-HOD antibody formation were examined.

RESULTS: Our results demonstrate that anti-HEL antibodies individually or in combination suppressed anti-HOD IgM, which correlated with the rate of detectable decrease in HEL on HOD RBCs. Furthermore, exposure to anti-HEL antibodies alone or in combination equally suppressed anti-HOD IgG formation. Unexpectedly, combination or individual anti-HEL antibodies induced AMIS and antigen modulation in an Fc γ R-independent manner. Pre-exposure of HOD RBCs to anti-HEL antibodies reduced antigen levels and suppressed anti-HOD antibody formation following HOD RBC exposure.

CONCLUSION: These results suggest that antibody-mediated antigen modulation may reflect a mechanism of AMIS that can occur independent of activating Fc γ Rs and may provide a surrogate to identify antibodies capable of inducing AMIS against different RBC antigens.

Antibodies to distinct red blood cell (RBC) antigens that develop following transfusion or pregnancy can lead to hemolytic disease of the fetus and newborn.^{1–4} Currently, the only commercially available prophylaxis that can actively prevent anti-RBC antibody development following antigen exposure is Rh immune globulin (RhIg).^{5–10} RhIg,

Address reprint requests to: Sean R. Stowell, MD, PhD, Center for Transfusion Medicine and Cellular Therapies, Department of Laboratory Medicine and Pathology, Emory University School of Medicine, 615 Michael Street, Atlanta, GA 30322; srstowe@emory.edu.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

polyclonal anti-RhD generated from pooled donor serum administered to prevent de novo anti-D development, is the most commonly employed example of antibody-mediated immunosuppression (AMIS). As the availability of RhIg donors is limited and the only available immunoprophylaxis is against RhD, there is a critical need to develop monoclonal antibody alternatives to RhIg and immunoprophylaxis to other RBC antigens. As different monoclonal antibodies to the RhD antigen can have differential immunological outcomes,^{11–20} understanding the mechanism regarding how monoclonal antibodies induce AMIS can facilitate the development and accurate assessment of monoclonal antibodies against RhD and other RBC antigens.

While the mechanisms responsible for RhIg-induced AMIS remain unknown, several hypotheses have been suggested, including rapid clearance and/or steric hindrance of RhD+ RBCs to prevent B-cell recognition or direct immune modulation.^{21–23} However, as RBC clearance rates do not necessarily correspond to the ability of an antibody to induce AMIS²³ and several studies suggest that steric hindrance or inhibition of the immune response through inhibitory Fc γ receptors (Fc γ Rs) may not play a role in AMIS,^{24–28} alternative mechanisms may be responsible. Recent studies using the KEL model system demonstrate that AMIS to the KEL RBC antigen correlates with antigen modulation,^{29,30} a phenomenon referring to antibody-induced loss of the target RBC antigen. In this setting, anti-KEL antibodies induce complement fixation and subsequent antigen modulation, which was found to be dependent on both complement and activating Fc γ Rs.^{31–36} Similarly, previous studies demonstrate that Fc γ Rs are also required for anti-HEL (hen egg lysozyme) antibody-induced antigen loss following engagement of the HEL antigen expressed in a membrane-bound form on RBCs (mHEL RBCs).³⁶ However, when HEL is expressed along with ovalbumin and human Duffy as a fusion protein on RBCs (HOD RBCs), AMIS to HOD occurs independent of Fc γ Rs,²⁸ suggesting that anti-HEL-induced AMIS following HOD RBC exposure may occur independent of antigen modulation. These collective data suggest that while antigen modulation may mediate anti-KEL-induced AMIS, anti-HEL antibodies likely induce AMIS to the HOD antigen through an alternative mechanism. Furthermore, while previous studies have separately examined monoclonal antibody-mediated AMIS to the HOD alloantigen and the role of Fc γ Rs in antigen modulation of HEL on mHEL RBCs,^{27,36,37} no studies have formally examined whether monoclonal anti-HELs antibodies mediate antigen modulation to the HOD antigen. Therefore, we sought to determine whether AMIS to HOD RBCs occurs via monoclonal antibody-induced antigen modulation.

To study this, we used the previously established HOD RBC model to define the impact of potential changes in surface RBC antigen as a mechanism responsible for AMIS.^{27,28,38,39} Our results demonstrate that monoclonal anti-HEL antibodies individually or in combination inhibit the downstream antibody response to HOD RBCs, in support of previous findings.²⁷ Anti-HEL-induced AMIS not only correlated with a decrease in the level of detectable HEL antigen on the RBC surface, but both antigen modulation and AMIS occurred independent of activating Fc γ R function. Together, these data suggest that monoclonal antibodies may inhibit antibody responses to some RBC antigens through Fc γ R-independent modulation of the target antigen.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice were purchased from Charles River and Fc γ R KO mice deficient in the common gamma chain were purchased from Taconic Laboratories. HOD transgenic mice were generated as previously described.³⁹ Emory Division of Animal Resources and Husbandry bred and housed the mice used in these experiments. Male and female mice were used at ages 8 to 12 weeks. The experimental protocols and animal procedures performed in these studies were approved by the Emory University Institutional Animal Care and Use Committee.

RBC labeling and transfusion

HOD+ and HOD- control RBCs were collected and labeled as previously described.^{37,38,40,41} Each recipient received approximately 10⁷ HOD+ RBCs (1:1 with HOD- RBCs) sensitized with 2.5 μ g/mL IgG1 monoclonal anti-HEL (clone: 2F4), 2.5 μ g/mL IgG1 monoclonal anti-HEL (clone: 4B7), or 1.25 μ g/mL of each monoclonal anti-HEL 2F4 and 4B7 in combination prior to transfusion, with phosphate-buffered saline alone as a control, as described previously.^{27,28}

RBC staining and antibody identification by flow cytometry

Following transfusion, mice were sampled at 10 minutes, 2 hours, and Days 1, 2, 3, and 5. For assessing detectable HEL antigen, RBCs were stained with 1:500 anti-HEL 2F4 and 4B7, followed by anti-mouse IgG (Jackson ImmunoResearch), as done previously.^{36,37,42} For the direct antiglobulin test (DAT), RBCs were stained with anti-mouse IgG. For complement component 3 detection, RBCs were stained with anti-mouse complement component 3 biotin (Cedarlane), followed by streptavidin (BD Biosciences).^{40,41} For detection of Duffy, RBCs were stained with 1:500 anti-Duffy monoclonal antibody, MIMA-29, followed by anti-mouse IgG. For detection of Ter-119, RBCs were stained with Ter-119 (BD Biosciences), as done previously.⁴⁰ To assess anti-HOD immunoglobulin M (IgM) and IgG antibody development, sera were flow cross-matched, as previously described.^{29,30,43-46} All antibodies were used at 1:100 in fluorescence activated cell sorting buffer (2% bovine serum albumin in phosphate-buffered saline) unless otherwise noted, as done previously.^{29,36,37,40-42,47,48}

Western blot analysis

RBCs isolated from each recipient group 2 days after immunization with anti-HEL monoclonal antibodies were lysed in 5 mmol/L sodium phosphate (pH 7.5) with protease inhibitor cocktail (Sigma Roche) at 4°C and centrifuged at 14,000 rpm, as outlined previously.^{29,48} RBC ghosts were then subjected to western blot analysis with rabbit polyclonal anti-HEL (Abcam, ab391, 1:5,000) and detection with goat anti-rabbit horseradish peroxidase (Abcam, ab205718 1:10,000). Mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (clone: GA1R, 1:1,000), followed by goat anti-mouse IgG1 horseradish peroxidase (Bethyl Laboratories, 1:10,000) was applied following stripping membranes with 25 mM glycine hydrochloride with 1% sodium dodecyl sulfate,

pH 2. All membranes were developed using a chemiluminescence reagent (HyGlo ECL, Denville Scientific Inc.).

Protease treatment

Following sensitization with anti-HEL monoclonals individually or in combination, HOD RBCs were incubated with decreasing concentrations of protease (Pronase, Sigma) starting from 2.5 µg/mL for 30 minutes at 37°C. Following protease treatment, RBCs were stained for HEL, Duffy, and Ter-119 antigens as outlined above.

Biotinylation

HOD mice were intravenously injected with 1 mg of reagent (EZ-Link Sulfo-NHS-Biotin, ThermoFisher Scientific). RBCs were stained with streptavidin (BD Biosciences) and evaluated for the levels of detectable HEL, Duffy, and Ter-119 as described.⁴⁰

Statistical analysis

Flow cytometry data was analyzed with computer software (FlowJo version 9.9.6, FlowJo, LLC) and statistical analysis was performed with biostatistics software (GraphPad Prism version 7, GraphPad Software). Three or more groups were compared by one-way analysis of variance with multiple comparisons by Tukey's test. For analysis of RBC survival and changes in detectable antigen following protease treatment, two-way analysis of variance with Dunnett's or Tukey's multiple comparisons test was utilized, respectively. A p value of less than 0.05 was the cutoff for significance.

RESULTS

Monoclonal anti-HEL antibodies decrease antibody formation and detectable antigen

We used a previously established model of AMIS wherein B6 recipients are exposed to HOD RBCs in the presence or absence of saturating concentrations of 2F4 and 4B7 anti-HEL monoclonal antibodies individually or in combination (Fig. 1A), each of which recognize distinct HEL epitopes (Fig. 1B).^{27,42} Consistent with previous studies in which anti-HOD IgM is detectable 3 to 5 days after transfusion of HOD RBCs and class switching to IgG occurring between days 7 and 14 after transfusion,^{43,44,49,50} exposure to HOD RBCs resulted in an anti-HOD IgM and IgG response, with no development of anti-HOD antibodies in HOD mice exposed to HOD RBCs (Fig. 1B). However, when HOD RBCs were transfused in the presence of anti-HEL antibodies, individually or in combination, the anti-HOD IgM response was blunted on Day 5 after transfusion (Fig. 1B). Furthermore, the anti-HOD IgG response was also suppressed in the presence of a combination or individual monoclonal antibodies (Fig. 1B). To determine whether antibody engagement differed over time, mice were exposed to labeled HOD RBCs, and RBCs were analyzed by a flow cytometry-based direct antiglobulin test (DAT) (Fig. 2A,B). The DAT revealed that IgG could be detected directly on HOD RBCs in the presence of anti-HEL antibodies at 10 minutes and 2 hours after transfusion (Fig. 2C and D). However, exposure to a combination of monoclonal anti-HEL antibodies resulted in a more rapid decrease in DAT levels than anti-HEL antibodies individually, with DAT levels decreasing through 5 days after transfusion (Fig. 2E-H).

While polyclonal antibodies have been shown to induce modulation of the HEL antigen on mHEL RBCs,^{36,41} monoclonal antibodies alone were not found to induce alterations to the target antigen,⁴² suggesting that the loss of detectable antibody on the HOD RBC surface over time may reflect antibody dissociation. To test this, the level of detectable antigen was examined over time (Fig. 3A). By 10 minutes and 2 hours after transfusion (Fig. 3B and C), the level of detectable HEL antigen was decreased in B6 mice exposed to HOD RBCs in the presence of individual or a combination of anti-HEL antibodies. Similar to the antibodies detected on the cell surface (Fig. 2), the level of HEL antigen following exposure to a combination of monoclonal antibodies was nearly undetectable. The level of HEL antigen on HOD RBCs in B6 mice exposed to monoclonal anti-HEL antibodies individually decreased over time when compared to HOD RBCs transfused into HOD mice (Fig. 3B–G). Loss of detectable antigen did not appear to reflect anti-HEL antibody–induced complement masking of the HEL antigen or RBC clearance, as no complement was detected on HOD RBCs when evaluated in parallel nor was clearance occurred regardless of anti-HEL exposure (Fig. 3H and I), consistent with previous results.^{36,42} Importantly, although the level of HEL antigen decreased by Day 5 after transfusion in B6 mice exposed to HOD RBCs in the absence of monoclonal anti-HEL antibodies, this HEL antigen decrease paralleled anti-HOD antibody development in the serum (Fig. 1),^{43,44,49,50} suggesting that development of *de novo* anti-HEL antibodies in B6 mice exposed to HOD RBCs may also induce changes in the level of detectable HEL antigen over time.

AMIS can occur independently of activating Fc γ R function

Given that our findings thus far indicate that monoclonal antibody-induced AMIS to HOD RBCs may occur as a result of changes in the level of detectable HEL antigen in B6 mice and that AMIS induced by antibody-mediated antigen modulation of the HEL antigen on mHEL RBCs occurs through an Fc γ R-mediated process,^{36,40} we tested the role of activating Fc γ Rs in monoclonal anti-HEL antibody–induced modulation of the HEL antigen on HOD RBCs. To investigate this, recipients deficient in activating Fc γ Rs (Fc γ R KO) were exposed to labeled HOD RBCs in the presence or absence of monoclonal anti-HEL antibodies, individually or in combination. In Fc γ R KO mice, HOD RBCs in the presence of monoclonal anti-HEL antibodies individually did not experience an initial decrease in their level of detectable HEL antigen at 10 minutes after transfusion (Fig. 4A), but began to display decreases in detectable antigen 2 hours after transfusion (Fig. 4B). By Day 1 after transfusion, HEL antigen levels on HOD RBCs in Fc γ R KO mice exposed to individual monoclonal antibodies began to significantly decline over time (Fig. 4C–F).

Similar to the decrease in detectable HEL antigen observed in B6 mice, reductions in HEL antigen levels in Fc γ R KO recipients exposed to HOD RBCs with monoclonal anti-HEL antibodies were also found to correlate with decreases in detectable antibody on the RBC surface. By 10 minutes after transfusion, IgG antibody was detectable on the HOD RBC surface in Fc γ R KO mice exposed to HOD RBCs in the presence of monoclonal anti-HEL antibodies, individually or in combination (Fig. 5A). However, from 2 hours through Day 5 after transfusion, the level of IgG antibody deposited on the HOD RBC surface decreased in Fc γ R KO mice that received HOD RBCs in the presence of monoclonal anti-HEL antibodies, individually or in combination (Fig. 5B–F). This decrease in bound IgG on the

HOD RBC surface paralleled the decrease in detectable HEL antigen (Fig. 4). Given the ability of monoclonal anti-HEL antibodies to decrease the level of detectable HEL antigen in B6 and Fc γ R KO mice, and as the decrease in antigen level correlated with AMIS in B6 mice (Figs. 1–4), we tested whether AMIS likewise occurred in Fc γ R KO mice. Exposure of Fc γ R KO mice to HOD RBCs in the absence of monoclonal anti-HEL antibodies resulted in the development of anti-HOD IgM and IgG at Days 5 and 14 after transfusion, respectively. However, exposure of Fc γ R KO mice to HOD RBCs in the presence of monoclonal anti-HEL antibodies resulted in equivalent inhibition of anti-HOD IgM and IgG formation (Fig. 6A). Similar to HOD RBC transfusion into B6 mice, monoclonal anti-HEL antibodies failed to fix detectable complement or alter HOD RBC clearance in Fc γ R KO mice (Fig. 6B and C).

Retransfusion of antigen-modulated HOD RBCs affects subsequent anti-HOD response

The dampened anti-HOD IgM response observed following exposure to monoclonal anti-HEL antibodies individually or in combination correlated with the decrease in detectable HEL antigen in B6 mice exposed to HOD RBCs in the presence of monoclonal anti-HEL antibodies, which may reflect early alterations in the availability of HEL antigen observed following exposure to anti-HEL antibodies individually or in combination. Homozygous and heterozygous HOD mice exhibit no detectable differences in HEL antigen expression (Fig. 7A), precluding direct examination of the potential impact of antigen density in the absence of antibody-induced changes in antigen levels. Therefore, to more directly test this, B6 mice were first exposed to HOD RBCs in the presence or absence of monoclonal anti-HEL antibodies to reach varying levels of antigen reduction. Using this approach, HOD RBCs were followed for changes in the level of detectable HEL antigen until a 50% or 90% reduction in HEL antigen on RBCs in the presence of monoclonal anti-HEL antibodies individually or in combination occurred (Fig. 7B), respectively, to approximate the level of detectable antigen observed on HOD RBCs 1 day after antibody exposure (Fig. 3D). RBCs were then harvested and re-transfused into naïve B6 recipients, followed by evaluation of the detectable HEL antigen over time to determine whether the HEL antigen further decreased in retransfused mice. At 10 minutes and Day 1 after transfusion, the level of detectable HEL antigen on retransfused HOD RBCs that were previously exposed to individual monoclonal anti-HEL antibodies remained stable (Fig. 7C and D). However, by Day 3 after retransfusion, the level of detectable HEL antigen decreased on the HOD RBCs that previously circulated in the presence of monoclonal anti-HEL antibodies individually (Fig. 7E). The level of detectable HEL antigen on HOD RBCs that were exposed to a combination of anti-HEL monoclonal antibodies remained low from 10 minutes through Day 5 after retransfusion (Fig. 7C–F). Re-transfusion of HOD RBCs with decreased antigen due to prior exposure to anti-HEL monoclonal antibodies individually or in combination resulted in an equally dampened anti-HOD response (Fig. 7G). The similar ability of individual or combinations of anti-HEL antibodies to induce AMIS did not appear to reflect differences in HOD RBC clearance following re-transfusion, as no change in HOD RBC clearance was observed following retransfusion (Fig. 7H).

HEL antigen decreases as HOD RBCs age

Given the ability of monoclonal antibodies to impact HEL antigen levels following HOD RBC exposure in Fc γ R KO mice, it remained possible that anti-HEL antibodies simply enhance an existing process of antigen removal on HOD RBCs. This is especially relevant when considering that antigens are not resynthesized on mature RBCs, and therefore certain RBC antigens may be more susceptible to degradation over time. Consistent with this, HEL antigen levels were consistently lower than the Duffy antigen on RBCs harvested from HOD mice (Fig. 8A), suggesting that HEL may be uniquely sensitive to degradation over time. To initially determine whether the HEL antigen may be more sensitive to proteolytic cleavage, HOD RBCs were incubated with a range of protease concentrations (Fig. 8B). HEL antigen displayed more sensitivity toward proteolytic cleavage than Duffy or Ter-119 antigens, which failed to experience similar changes in levels under the same conditions (Fig. 8C–E). However, inclusion of anti-HEL antibodies failed to alter the differential sensitivity of HEL to proteolytic cleavage (Fig. 8B–E). As a result, we next sought to simply determine whether HEL is selectively lost over time on HOD RBCs as they age *in vivo* in the absence of anti-HEL antibodies. To accomplish this, HOD RBC donor mice were biotinylated, followed by tracking HOD RBCs over time (Fig. 8F). While biotinylation itself failed to impact the level of detectable HEL, Duffy, or Ter-119, by 14 days after biotinylation, the level of HEL decreased. In contrast, similar changes failed to occur in Duffy or Ter-119 when evaluated in parallel (Fig. 8G–I). Despite the inability of anti-HEL antibodies to impact HEL antigen sensitivity to proteolytic cleavage *in vitro*, we next sought to determine whether selective loss of HEL likewise occurs following antibody-mediated HEL antigen removal (Fig. 8J). HEL antigen on HOD RBCs experienced a 50% or 90% decrease in the presence of monoclonal anti-HEL antibodies, individually or in combination, respectively (Fig. 8K) with no detectable change in Duffy antigen (Fig. 8L). Despite the absence of detectable complement fixation on the RBC surface (Fig. 3H), it remained possible that antibody fragments may mask the HEL antigen, resulting in apparent HEL antigen loss. To test this, western blot analysis was performed. Using this approach, HEL antigen loss was observed following exposure to monoclonal anti-HEL antibodies individually or in combination (Fig. 8M). Taken together, these results suggest that anti-HEL antibodies may accelerate an existing process of HEL antigen removal.

DISCUSSION

In this study, monoclonal anti-HEL antibodies were not only found to correlate with a dampened IgM antibody response to the HOD RBC antigen, but also a decreased level of detectable HEL antigen. These findings suggest that monoclonal antibodies may induce AMIS by decreasing detectable HEL antigen levels below the threshold necessary to activate HEL-reactive B cells. B cell activation typically requires not only a threshold concentration of antigen, but also a threshold of receptor stimulation.^{51–56} Consistent with this, lowering the level of detectable HEL antigen by 50% resulted in an equivalent degree of IgM suppression. As no difference in the anti-HOD IgG response was observed whether HOD RBCs were transfused in the presence of monoclonal anti-HEL antibodies alone or in combination, the similar level of IgG suppression suggests that by the time class switching occurs, HEL antigen levels may have dropped below the threshold needed to efficiently

induce anti-HEL IgG. As suppression of IgG antibody formation is the primary target of immunoprophylaxis, these results suggest that monoclonal IgG antibodies that initially only partially induce antigen loss may be sufficient for equivalent inhibition of IgG formation.

As the consequence of antibody-antigen interactions can differ, the requirements of AMIS for a particular antigen may likewise uniquely depend on the target antigen. For example, the reduced efficacy of monoclonal antibodies to induce AMIS toward RhD may in part reflect a need for multiple antibodies to induce antigen modulation,^{24,58} as demonstrated for HEL antigen expression on mHEL RBCs.⁴² Although recent studies demonstrate that anti-RhD can induce antigen modulation clinically,⁵⁹ it is certainly possible that for RhD and possibly other antigens, a combination of RBC clearance and antigen modulation collectively contribute to AMIS. As anti-RhD represents the only clinically available AMIS intervention, understanding potential differences in the outcome of antibody engagement of a variety of RBC antigens is important in understanding both common and distinct features of antibody-antigen interactions that may result in AMIS.^{28,29,36–38,40–42,47,48,57} Such information will likely prove vital for rational approaches to be developed to target other antigens for which no immunoprophylaxis currently exists.

In addition to a potential threshold of detectable HEL antigen necessary to induce a productive anti-HOD antibody response, the rate at which the HEL antigen is modulated may likewise impact the development of anti-HOD antibodies. Given that HOD RBCs in the presence of monoclonal anti-HEL antibodies undergo a more rapid decrease in the level of detectable HEL antigen than HOD RBCs transfused into B6 recipients in the absence of monoclonal anti-HEL antibodies, the removal of antigen at early time points may prevent sufficient detection and therefore response to HOD at these earlier time points. Actual differences or trends toward reduced ability of individual monoclonal antibodies to suppress anti-HOD IgM may likewise reflect distinct windows wherein antigen substrate is needed for anti-HOD IgM formation in contrast to the possibility of slightly later antigen requirements for IgG antibody formation. While the ability of anti-HEL antibodies to modulate antigen suggests that such thresholds exist, the exact threshold requirements for productive antibody formation remain unknown. Unfortunately, as antibody provided the only mechanism of selectively removing HEL from HOD RBCs, we cannot rule out the potential impact of antibody remaining on the cell surface on antibody formation. While proteolytic cleavage allowed HEL to be selectively removed in vitro, many proteins are sensitive to protease,⁶⁰ which could impact RBC circulatory half-life, interactions with immune cells or other RBC changes that may influence the overall immune response to HOD, precluding the use of this approach to examine the potential impact of HEL antigen levels on anti-HOD formation. HOD founders that express different levels of HOD, as recently described for KEL,⁴⁶ will ultimately be needed to begin defining potential antigen threshold requirements for a productive immune response against the HOD antigen.

While antigen removal still occurred in Fc γ R KO recipients, the rate of antigen modulation was reduced compared to B6 mice, suggesting that while antigen modulation and AMIS can occur independent of Fc γ Rs, these receptors may contribute to early antigen removal. Consistent with this, previous studies indicate that Fc γ Rs can facilitate antigen removal.^{40,41} However, given that previous studies have refuted the involvement of both activating and

inhibitory Fc γ Rs in polyclonal antibody-mediated AMIS to HOD RBCs,²⁸ and our study shows that the monoclonal anti-HEL antibodies do not fix complement nor are activating Fc γ Rs required for AMIS or antigen modulation to occur, these studies therefore suggest a novel mechanism of antigen removal distinct from those previously described.^{29,41} Similarly, while monoclonal anti-HEL antibodies individually or in combination induced AMIS equivalently in Fc γ R KO mice for both IgM and IgG, unimmunized Fc γ R KO mice may potentially develop a decreased antibody response to HOD RBCs compared to B6 mice, indicating that Fc γ Rs may be involved in other aspects of anti-HOD immunity.^{61–65} As a result, while Fc γ Rs are dispensable for AMIS in this model, they may impact anti-HOD formation more generally.

As antigen is removed on HOD RBCs as RBCs age over time, despite no difference in HEL antigen levels *in vitro* following incubation of HOD RBCs with anti-HEL antibodies in the presence or absence of proteases, endothelial-derived or other organ resident proteases, in addition to shear forces, may cumulatively impact HEL antigen levels following antibody engagement *in vivo*. Given that ligand has been shown to activate platelets via proteolytic cleavage of cell surface proteins in a shear-dependent manner,⁶⁶ it is possible that shear forces may potentially work synergistically with antibody to alter HOD cleavage site accessibility. In this setting, nonclassical type II Fc receptors outside the type I Fc γ R family, including CD23 and possible other lectin receptors,⁶⁷ may engage IgG, facilitating shear-dependent forces as antibody is tethered to the RBC through the HEL antigen. Similar mechanisms may be involved in the apparent ability of the endogenous immune response to likewise impact HEL antigen levels in the absence of monoclonal anti-HEL antibodies. However, it is important to note that increased sensitivity of HEL to proteolytic cleavage *in vitro* may not reflect antibody-induced loss of the HEL antigen *in vivo*, which may instead represent an entirely different process. Distinct mechanisms of antigen modulation may play unique roles in different antigen systems, each of which may be differentially sensitive to variables such as saturation, involvement of antibody effector systems and level of antibody antigen engagement required. Although antibody-induced antigen loss has been documented in various settings in both animal models and humans,^{30,40,48,59,68,69} the underlying mechanisms whereby these processes occur remain to be determined.

As with all experimental models, limitations must be considered. In this study, we examine one particular RBC antigen in combination with two monoclonal anti-HEL antibodies previously validated to induce AMIS in this model system.²⁷ Therefore, while both monoclonal anti-HEL antibodies tested induced antigen modulation and recent studies demonstrate that monoclonal antibodies that target RBC antigens can induce antigen loss in patients,^{48,59} whether antigen modulation is a general phenomenon that occurs following antibody engagement of other RBC antigens remains to be determined. Furthermore, despite lack of analysis of Duffy antigen by western blot analysis, loss of the HEL antigen by western blot analysis, coupled with similar loss of HEL detection by flow cytometric analysis in the absence of similar changes in Duffy antigen levels, suggests that anti-HEL antibodies induce selective antigen removal. While pre-clinical models possess limitations, these models allow us to investigate potential molecular mechanisms whereby general principles of AMIS may occur. As the KEL and HOD models are the only systems published to date that render themselves to AMIS studies, these findings suggest that antigen

modulation may play a role in AMIS. However, whether this approach actually prevents alloimmunization against other RBC antigens remains to be tested. Using this and related model systems may therefore provide important insight when seeking to answer questions in transfusion medicine surrounding the development of antibodies capable of inducing AMIS.

ACKNOWLEDGMENTS

AM and SRS designed experiments. SRP and CMA provided technical and intellectual guidance on experimental design, troubleshooting, and manuscript preparation. AM performed all experiments and analyzed data. AM and SRS wrote the manuscript.

Supported, in part, by the NIH NHLBI R01 HL13557, NHLBI R01 HL138656, NHLBI P01 HL132819, NIH Director's Early Independence Award Program (DP5OD019892) and the Burroughs Wellcome Trust Career Award to Medical Scientists to SRS.

ABBREVIATIONS:

AMIS	antibody-mediated immunosuppression
DAT	direct antiglobulin test
FcγRs	Fc γ receptors
HEL	hen egg lysozyme
HOD	hen egg lysozyme-ovalbumin-Duffy
mHEL	membrane-bound form of HEL

REFERENCES

1. Stowell SR, Henry KL, Smith NH, et al. Alloantibodies to a paternally derived RBC KEL antigen lead to hemolytic disease of the fetus/newborn in a murine model. *Blood* 2013;122: 1494–504. [PubMed: 23801629]
2. Heard DH, Hinde IT, Mynors LS. An experimental study of haemolytic disease of the newborn due to isoimmunization of pregnancy. *J Hyg* 1949;47:119–31. [PubMed: 15392612]
3. Moise KJ. Fetal anemia due to non-Rhesus-D red-cell alloimmunization. *Semin Fetal Neonatal Med* 2008;13:207–14. [PubMed: 18396474]
4. Moise KJ Jr. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2008;112:164–76. [PubMed: 18591322]
5. Clarke CA, Finn R, Lehane D, et al. Dose of anti-D gamma-globulin in prevention of Rh-haemolytic disease of the new- born. *Br Med J* 1966;1:213–4. [PubMed: 4159118]
6. Finn R, Clarke CA, Donohoe WT, et al. Experimental studies on the prevention of Rh haemolytic disease. *Br Med J* 1961;1: 1486–90. [PubMed: 13699661]
7. Clarke CA, Donohoe WT, McConnell R, et al. Further experimental studies on the prevention of Rh haemolytic disease. *Br Med J* 1963;1:979–84. [PubMed: 14021558]
8. Pollack W, Gorman JG, Freda VJ, et al. Results of clinical trials of RhoGAM in women. *Transfusion* 1968;8:151–3. [PubMed: 4173363]
9. Freda VJ, Gorman JG, Pollack W. Rh factor: prevention of isoimmunization and clinical trial on mothers. *Science* 1966;151: 828–30. [PubMed: 17746731]
10. Freda VJ, Gorman JG, Pollack W. Successful prevention of experimental Rh sensitization in man with an anti-Rh gamma2-globulin antibody preparation: a preliminary report. *Transfusion* 1964;4:26–32. [PubMed: 14105934]

11. Walker MR, Kumpel BM, Thompson K, et al. Immunogenic and antigenic epitopes of immunoglobulins binding of human monoclonal anti-D antibodies to FcRI on the monocyte-like U937 cell line. *Vox Sang* 1988;55:222–8. [PubMed: 2464239]
12. Kumpel BM, Leader KA, Poole GD, et al. Human and murine anti-Rh monoclonal antibodies. A comparison of serological, immunochemical, cellular and functional activity. *Rev Fr Transfus Immunohematol* 1988;31:187–201. [PubMed: 3145548]
13. Wiener E, Jolliffe VM, Scott HC, et al. Differences between the activities of human monoclonal IgG1 and IgG3 anti-D antibodies of the Rh blood group system in their abilities to mediate effector functions of monocytes. *Immunology* 1988;65: 159–63. [PubMed: 3192267]
14. Hadley AG, Kumpel BM. Phagocytosis by human monocytes of red cells sensitized with monoclonal IgG1 and IgG3 anti-D. *Vox Sang* 1989;57:150–1. [PubMed: 2506695]
15. Kumpel BM, Wiener E, Urbaniak SJ, et al. Human monoclonal anti-D antibodies. II. The relationship between IgG subclass, Gm allotype and Fc mediated function. *Br J Haematol* 1989;71:415–20. [PubMed: 2539182]
16. Kumpel BM, Leader KA, Merry AH, et al. Heterogeneity in the ability of IgG1 monoclonal anti-D to promote lymphocyte-mediated red cell lysis. *Eur J Immunol* 1989;19:2283–8. [PubMed: 2481589]
17. Kumpel BM, Hadley AG. Functional interactions of red cells sensitized by IgG1 and IgG3 human monoclonal anti-D with enzyme-modified human monocytes and FcR-bearing cell lines. *Mol Immunol* 1990;27:247–56. [PubMed: 2111455]
18. Bakacs T, Kumpel BM, Tusnady G. Haemolysis mediated by anti-D monoclonal antibodies in direct and cold target competition ADCC assays. *Immunol Lett* 1992;32:223–31. [PubMed: 1500093]
19. Hadley AG, Zupanska B, Kumpel BM, et al. The functional activity of Fc gamma RII and Fc gamma RIII on subsets of human lymphocytes. *Immunology* 1992;76:446–51. [PubMed: 1356095]
20. Kumpel BM, Goodrick MJ, Pamphilon DH, et al. Human Rh D monoclonal antibodies (BRAD-3 and BRAD-5) cause accelerated clearance of Rh D+ red blood cells and suppression of Rh D immunization in Rh D- volunteers. *Blood* 1995;86:1701–9. [PubMed: 7655002]
21. Brinc D, Lazarus AH. Mechanisms of anti-D action in the prevention of hemolytic disease of the fetus and newborn. *Hematol Am Soc Hematol Educ Program* 2009;2009:185–91.
22. Brinc D, Le-Tien H, Crow AR, et al. IgG-mediated immunosuppression is not dependent on erythrocyte clearance or immunological evasion: implications for the mechanism of action of anti-D in the prevention of haemolytic disease of the newborn? *Br J Haematol* 2007;139:275–9. [PubMed: 17897303]
23. Kumpel BM, Elson CJ. Mechanism of anti-D-mediated immune suppression - a paradox awaiting resolution? *Trends Immunol* 2001;22:26–31. [PubMed: 11286688]
24. Kumpel BM. Efficacy of RhD monoclonal antibodies in clinical trials as replacement therapy for prophylactic anti-D immunoglobulin: more questions than answers. *Vox Sang* 2007;93: 99–111. [PubMed: 17683353]
25. Brinc D, Denomme GA, Lazarus AH. Mechanisms of anti-D action in the prevention of hemolytic disease of the fetus and newborn: what can we learn from rodent models? *Curr Opin Hematol* 2009;16:488–96. [PubMed: 19730101]
26. Bergstrom JJ, Heyman B. IgG suppresses antibody responses in mice lacking C1q, C3, complement receptors 1 and 2, or IgG Fc-receptors. *PLoS One* 2015;10:e0143841. [PubMed: 26619292]
27. Bernardo L, Amash A, Marjoram D, et al. Antibody-mediated immune suppression is effective when blends of anti-RBC monoclonal antibodies are used in mice. *Blood* 2016;128: 1076–80. [PubMed: 27330002]
28. Bernardo L, Yu H, Amash A, et al. IgG-mediated immune suppression to erythrocytes by polyclonal antibodies can occur in the absence of activating or inhibitory Fcγ receptors in a full mouse model. *J Immunol* 2015;195:2224–30. [PubMed: 26188060]
29. Liu J, Santhanakrishnan M, Natarajan P, et al. Antigen modulation as a potential mechanism of anti-KEL immunoprophylaxis in mice. *Blood* 2016;128:3159–68. [PubMed: 27688803]

30. Stowell SR, Arthur CM, Girard-Pierce KR, et al. Anti-KEL sera prevents alloimmunization to transfused KEL RBCs in a murine model. *Haematologica* 2015;100:e394–7. [PubMed: 26185172]
31. Powers A, Mohammed M, Uhl L, et al. Apparent nonhemolytic alloantibody- induced red-cell antigen loss from transfused erythrocytes. *Blood* 2007;109:4590. [PubMed: 17483302]
32. Williamson LM, Poole J, Redman C, et al. Transient loss of proteins carrying Kell and Lutheran red cell antigens during consecutive relapses of autoimmune thrombocytopenia. *Br J Haematol* 1994;87:805–12. [PubMed: 7527243]
33. Vengelen-Tyler V, Gonzalez B, Garratty G, et al. Acquired loss of red cell Kell antigens. *Br J Haematol* 1987;65:231–4. [PubMed: 3828231]
34. Seyfried H, Gorska B, Maj S, et al. Apparent depression of antigens of the Kell blood group system associated with autoimmune acquired haemolytic anaemia. *Vox Sang* 1972;23: 528–36. [PubMed: 4657687]
35. Beck ML, Marsh WL, Pierce SR, et al. Auto anti-Kpb associated with weakened antigenicity in the Kell blood group system: a second example. *Transfusion* 1979;19:197–202. [PubMed: 86215]
36. Zimring JC, Hair GA, Chadwick TE, et al. Nonhemolytic antibody-induced loss of erythrocyte surface antigen. *Blood* 2005;106:1105–12. [PubMed: 15831698]
37. Yu H, Stowell SR, Bernardo L, et al. Antibody-mediated immune suppression of erythrocyte alloimmunization can occur independently from red cell clearance or epitope masking in a murine model. *J Immunol* 2014;193:2902–10. [PubMed: 25122924]
38. Liepkalns JS, Hod EA, Stowell SR, et al. Biphasic clearance of incompatible red blood cells through a novel mechanism requiring neither complement nor Fc γ receptors in a murine model. *Transfusion* 2012;52:2631–45. [PubMed: 22502635]
39. Desmarests M, Cadwell CM, Peterson KR, et al. Minor histo-compatibility antigens on transfused leukoreduced units of red blood cells induce bone marrow transplant rejection in a mouse model. *Blood* 2009;114:2315–22. [PubMed: 19525479]
40. Stowell SR, Liepkalns JS, Hendrickson JE, et al. Antigen modulation confers protection to red blood cells from antibody through Fc γ receptor ligation. *J Immunol* 2013;191: 5013–25. [PubMed: 24108700]
41. Girard-Pierce KR, Stowell SR, Smith NH, et al. A novel role for C3 in antibody-induced red blood cell clearance and antigen modulation. *Blood* 2013;122:1793–801. [PubMed: 23878139]
42. Zimring JC, Cadwell CM, Chadwick TE, et al. Nonhemolytic antigen loss from red blood cells requires cooperative binding of multiple antibodies recognizing different epitopes. *Blood* 2007;110:2201–8. [PubMed: 17569819]
43. Hendrickson JE, Hod EA, Perry JR, et al. Alloimmunization to transfused HOD red blood cells is not increased in mice with sickle cell disease. *Transfusion* 2012;52:231–40. [PubMed: 21790627]
44. Stowell SR, Smith NH, Zimring JC, et al. Addition of ascorbic acid solution to stored murine red blood cells increases post-transfusion recovery and decreases microparticles and alloimmunization. *Transfusion* 2013;53:2248–57. [PubMed: 23384196]
45. Arthur CM, Patel SR, Sullivan HC, et al. CD8+ T cells mediate antibody-independent platelet clearance in mice. *Blood* 2016; 127:1823–7. [PubMed: 26787734]
46. Arthur CM, Patel SR, Smith NH, et al. Antigen density dictates immune responsiveness following red blood cell transfusion. *J Immunol* 2017;198:2671–80. [PubMed: 28250159]
47. Liepkalns JS, Cadwell CM, Stowell SR, et al. Resistance of a subset of red blood cells to clearance by antibodies in a mouse model of incompatible transfusion. *Transfusion* 2013;53: 1319–27. [PubMed: 23033973]
48. Sullivan HC, Gerner-Smidt C, Nooka AK, et al. Daratumumab (anti-CD38) induces loss of CD38 on red blood cells. *Blood* 2017;129:3033–7. [PubMed: 28373263]
49. Hendrickson JE, Hod EA, Spitalnik SL, et al. Storage of murine red blood cells enhances alloantibody responses to an erythroid-specific model antigen. *Transfusion* 2010;50:642–8. [PubMed: 19906034]
50. Calabro S, Gallman A, Gowthaman U, et al. Bridging channel dendritic cells induce immunity to transfused red blood cells. *J Exp Med* 2016;213:887–96. [PubMed: 27185856]
51. Carter RH, Fearon DT. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* 1992;256: 105–7. [PubMed: 1373518]

52. Depoil D, Fleire S, Treanor BL, et al. CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand. *Nat Immunol* 2008;9:63–72. [PubMed: 18059271]
53. Dempsey PW, Allison ME, Akkaraju S, et al. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 1996;271:348–50. [PubMed: 8553069]
54. Fleire SJ, Goldman JP, Carrasco YR, et al. B cell ligand discrimination through a spreading and contraction response. *Science* 2006;312:738–41. [PubMed: 16675699]
55. Sato S, Miller AS, Howard MC, et al. Regulation of B lymphocyte development and activation by the CD19/CD21/CD81/Leu 13 complex requires the cytoplasmic domain of CD19. *J Immunol* 1997;159:3278–87. [PubMed: 9317126]
56. Adelstein S, Pritchard-Briscoe H, Anderson TA, et al. Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen. *Science* 1991;251:1223–5. [PubMed: 1900950]
57. Cruz-Leal Y, Marjoram D, Lazarus AH. Erythrocyte saturation with IgG is required for inducing antibody-mediated immune suppression and impacts both erythrocyte clearance and antigen-modulation mechanisms. *J Immunol* 2018;200: 1295–305. [PubMed: 29358275]
58. Beliard R. Monoclonal anti-D antibodies to prevent alloimmunization: lessons from clinical trials. *Transfus Clin Biol* 2006;13: 58–64. [PubMed: 16580863]
59. Sullivan HC, Arthur CM, Thompson L, et al. Anti-RhD reduces levels of detectable RhD antigen following anti-RhD infusion. *Transfusion* 2018;58:542–4. [PubMed: 29411397]
60. Pourazar A. Red cell antigens: structure and function. *Asian J Transfus Sci* 2007;1:24–32. [PubMed: 21938229]
61. Ravetch JV. Fc receptors: rubor redux. *Cell* 1994;78:553–60. [PubMed: 8069908]
62. Fanger NA, Voigtlaender D, Liu C, et al. Characterization of expression, cytokine regulation, and effector function of the high affinity IgG receptor Fc gamma RI (CD64) expressed on human blood dendritic cells. *J Immunol* 1997;158:3090–8. [PubMed: 9120261]
63. Regnault A, Lankar D, Lacabanne V, et al. Fcγ receptor- mediated induction of dendritic cell maturation and major histo-compatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* 1999;189: 371–80. [PubMed: 9892619]
64. Amigorena S, Salamero J, Davoust J, et al. Tyrosine-containing motif that transduces cell activation signals also determines internalization and antigen presentation via type III receptors for IgG. *Nature* 1992;358:337–41. [PubMed: 1386408]
65. Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol* 2001;19:275–90. [PubMed: 11244038]
66. Quach ME, Dragovich MA, Chen W, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. *Blood* 2018;131:787–96. [PubMed: 29203584]
67. Pincetic A, Bournazos S, DiLillo DJ, et al. Type I and type II Fc receptors regulate innate and adaptive immunity. *Nat Immunol* 2014;15:707–16. [PubMed: 25045879]
68. Taylor RP, Lindorfer MA. Antigenic modulation and rituximab resistance. *Semin Hematol* 2010;47:124–32. [PubMed: 20350659]
69. Beum PV, Lindorfer MA, Taylor RP. Within peripheral blood mononuclear cells, antibody-dependent cellular cytotoxicity of rituximab-opsonized Daudi cells is promoted by NK cells and inhibited by monocytes due to shaving. *J Immunol* 2008;181: 2916–24. [PubMed: 18684983]

Fig. 1.

Anti-HEL monoclonal antibodies saturate HOD RBCs and dampen anti-HOD antibody response. (A) Antibody saturation of HOD RBCs individually or in combination was assessed by flow cytometry. (B) Following exposure of B6 mice to HOD RBCs in the presence (2F4, 4B7, or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of anti-HEL monoclonal antibodies, serum was assessed for the presence of anti-HOD IgM on Day 5 and anti-HOD IgG on Day 14 after transfusion by flow cross-match. Following exposure of HOD mice to HOD RBCs, anti-HOD IgM and IgG was assessed on Days 5 and 14 after transfusion, respectively, by flow-crossmatch. For B, **** = $p < .0001$, *** = $p < .0002$ and * = $p < .05$ by one-way analysis of variance with Tukey's multiple comparisons test. Means $\pm$ SD shown. HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; IgG = immunoglobulin G; IgM = immunoglobulin M; RBCs = red blood cells.

Fig. 2.

Anti-HEL monoclonal antibodies are detected on HOD RBCs following transfusion. (A) B6 mice were exposed to Dil-labeled HOD + RBCs and control DiO-labeled HOD RBCs in the presence (2F4, 4B7, or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of anti-HEL monoclonal antibodies. (B) The deposition of surface IgG antibody specifically on HOD+ RBCs was probed at 10 minutes (C), 2 hours (D), Day 1 (E), Day 2 (F), Day 3 (G) and Day 5 (H). For C-H, **** = $p < .0001$, *** = $p < .0008$, ** = $p < .008$, and * = $p < .05$ by one-way analysis of variance with Tukey's multiple comparisons test. Means $\pm$ SD shown. HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; IgG = immunoglobulin G; RBCs = red blood cells.

Fig. 3.

Anti-HEL antibodies induce a decrease in the level of detectable HEL antigen and fail to deposit complement or induce clearance. (A) B6 mice were exposed to a mixture of DiI-labeled HOD+ RBCs and control DiO-labeled HOD- RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of monoclonal anti-HEL antibodies. (B-G) Following transfusion, the level of HEL antigen on circulating HOD+ RBCs was assessed at 10 minutes (B), 2 hours (C), Day 1 (D), Day 2 (E), Day 3 (F), and Day 5 (G). Level of HEL antigen was measured by percentage of HEL antigen normalized to the HOD control group. (H) HOD + RBCs in the absence (phosphate-buffered saline) or presence of monoclonal anti-HEL antibodies (2F4 and 4B7) individually or in combination (combo) were examined for complement component 3 deposition following exposure in B6 mice at 10 minutes and 2 hours after transfusion. (I) Clearance of HOD+ RBCs in B6 mice exposed to DiI-labeled HOD+ RBCs and control DiO-labeled HOD- RBCs in the presence or absence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo). For B-G, **** = $p < .0001$, *** = $p < .0006$, ** = $p < .002$ and * = $p < .05$ by One-way analysis of variance with Tukey's multiple comparisons test. For I, statistical analysis was performed using two-way analysis of variance with

Dunnett's multiple comparisons test. Means $\pm$ SD shown. HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; RBCs = red blood cells.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4.

Anti-HEL antibodies induce a decrease in the level of detectable HEL antigen in Fc γ R KO mice. (A-F) Fc γ R KO mice (Fc γ) were exposed to a mixture of Dil-labeled HOD+ RBCs and control DiO-labeled HOD- RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of monoclonal anti-HEL antibodies. RBCs were collected and the percentage of detectable HEL antigen, normalized to control HOD mice exposed to HOD+ RBCs, was measured at 10 minutes (A), 2 hours (B), Day 1 (C), Day 2 (D), Day 3 (E), and Day 5 (F). For A-F, **** = $p < .0001$, *** = $p < .003$, ** = $p < .009$ and * = $p < .05$ by one-way analysis of variance with Tukey's multiple comparisons test. Means $\pm$ SD shown. Fc γ R = Fc γ receptor; HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; RBCs = red blood cells.

Fig. 5.

HOD RBCs exposed to anti-HEL antibodies display decreased levels of bound antibody in FcγR KO mice. (A-F) FcγR KO mice (Fcγ) were exposed to a mixture of DiI-labeled HOD + RBCs and control DiO-labeled HOD- RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of monoclonal anti-HEL antibodies. Following transfusion, RBCs were assessed for IgG deposition via the direct antiglobulin test at 10 minutes (A), 2 hours (B), Day 1 (C), Day 2 (D), Day 3 (E) and Day 5 (F). For A-F, **** = $p < 0.0001$, *** = $p < .0006$, ** = $p < .002$ and * = $p < .05$ by one-way analysis of variance with Tukey's multiple comparisons test. Means $\pm$ SD shown. FcγR = Fcγ receptor; HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; IgG = immunoglobulin G; RBCs = red blood cells.

Fig. 6.

HOD RBCs exposed to anti-HEL antibodies in FcγR KO mice results in dampening of the anti-HOD antibody response without complement deposition nor clearance of HOD RBCs. (A) Serum was assessed for the presence of anti-HOD IgM on Day 5 and anti-HOD IgG on Day 14 after transfusion of HOD+ RBCs in FcγR KO mice (Fcγ) by flow cross-match. (B) HOD RBCs in the absence (phosphate-buffered saline) or presence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo) were examined for C3 deposition following exposure in FcγR KO mice (Fcγ) at 10 minutes and 2 hours after transfusion. (C) Clearance of HOD RBCs in FcγR KO mice (Fcγ) exposed to DiI-labeled HOD+ RBCs and control DiO-labeled HOD- RBCs in the presence or absence of anti-HEL monoclonal antibodies (2F4 and 4B7) individually or in combination (combo). For A, **** = $p < .0001$, *** = $p < .0003$ and ** = $p < .003$ by one-way analysis of variance with Tukey's multiple comparisons test. For C, statistical analysis was performed using two-way analysis of variance with Dunnett's multiple comparisons test. Means $\pm$ SD shown. FcγR = Fcγ receptor; HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; IgG = immunoglobulin G; IgM = immunoglobulin M; RBCs = red blood cells. [Color figure can be viewed at wileyonlinelibrary.com]

Fig. 7.

Re-transfusion of HOD RBCs with varying levels of HEL antigen correlates with dampening of the anti-HOD antibody response without clearance of HOD RBCs. (A) HEL antigen on homozygous and heterozygous HOD mice was assessed by flow cytometry. (B) B6 mice were exposed to HOD+ RBCs in the presence (2F4, 4B7 or a combination of 2F4 and 4B7 [combo]) or absence (phosphate-buffered saline) of monoclonal anti-HEL antibodies. The level of detectable HEL antigen was then assessed followed by re-transfusion of RBCs into naive B6 recipients. (C-F) Following re-transfusion, the level of HEL antigen was detected at 10 minutes (C), Day 1 (D), Day 3 (E), and Day 5 (F). (G) Serum was collected from retransfused recipients, and the development of anti-HOD IgM and IgG was assessed on Day 5 and Day 14 after transfusion, respectively, by flow cross-match. (H) Clearance of anti-HEL monoclonal antibody-antigen modulated HOD+ RBCs following re-transfusion into naive B6 mice. For B-G, **** = $p < .0001$, *** = $p < .0002$ and * = $p < .05$ significant by one-way analysis of variance with Tukey's multiple comparisons test. For H, statistical analysis was performed using two-way analysis of variance with Dunnett's multiple comparisons test. Means $\pm$ SD shown. HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; IgG = immunoglobulin G; IgM =

immunoglobulin M; RBCs = red blood cells. [Color figure can be viewed at wileyonlinelibrary.com]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 8.

HEL antigen decreases over time on HOD RBCs as they age but not with protease treatment *in vitro*. (A) HOD+ RBCs stained for the level of detectable HEL and Duffy antigens, followed by overlay of histograms. (B) HOD+ RBCs were treated with proteases in the presence or absence of monoclonal anti-HEL antibodies. Following treatment, the level of detectable HEL (C), Duffy (D), and Ter-119 (E) antigens were measured by flow cytometry. (F) HOD mice were biotinylated via intravenous injection of NHS-sulfo-biotin, followed by tracking biotin positive RBCs in vivo over time. (G-I) RBCs were harvested and evaluated for levels of HEL (G), Duffy (H), and Ter-119 (I) by flow cytometry on Day 0 (D0) or Day 14 (D14) after biotinylation. (J) B6 mice were either not immunized (phosphate-buffered saline) or passively immunized with anti-HEL monoclonal antibodies (2F4, 4B7 or a combination of 2F4 and 4B7 [combo]), followed by exposure to HOD+ RBCs. Two days after transfusion, HOD+ RBCs were harvested. The level of detectable HEL antigen (K) and

Duffy antigen (L) were then measured by flow cytometry. RBCs were also subjected to western blot analysis for HEL (M). For C-E, statistical analysis was performed using two-way analysis of variance with Tukey's multiple comparison's test. For G-L, **** = $p < .0001$ and *** = $p < .0003$ by one-way analysis of variance with Tukey's multiple comparisons test. Means $\pm$ SD shown. HEL = hen egg lysozyme; HOD = hen egg lysozyme-ovalbumin-Duffy; RBCs = red blood cells.