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*J Immunol* published online 9 October 2013
http://www.jimmunol.org/content/early/2013/10/08/jimmunol.1300885

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/10/09/jimmunol.1300885.DC1

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Antigen Modulation Confers Protection to Red Blood Cells from Antibody through Fcγ Receptor Ligation

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Autoantibodies and alloantibodies can damage self-tissue or transplanted tissues through either fixation of complement or ligation of FcγRs. Several pathways have been described that imbue self-tissues with resistance to damage from complement fixation, as a protective measure against damage from these Abs. However, it has been unclear whether parallel pathways exist to provide protection from FcγR ligation by bound Abs. In this article, we describe a novel pathway by which cell surface Ag is specifically decreased as a result of Ab binding (Ag modulation) to the extent of conferring protection to recognized cells from Fcγ-dependent clearance. Moreover, the Ag modulation in this system requires FcγR ligation. Together, these findings provide unique evidence of self-protective pathways for FcγR-mediated Ab damage. The Journal of Immunology, 2013, 191: 000–000.

Although adaptive immunity has the capacity to recognize a vast range of antigenic determinants, this inherent plasticity also increases the risk of autoimmunity (1). As a result, a distinct set of regulatory mechanisms evolved to aid adaptive immunity in discriminating self from non-self (2, 3). Although central and peripheral tolerance mechanisms reduce the likelihood of self-reactivity, these regulatory programs occasionally fail to fully eliminate or inactivate self-reactive cells (4). When tolerance mechanisms fail, the cellular targets of immunity themselves can provide an additional layer of protection through resistance to effector mechanisms. Such cellular protection often reflects cell surface expression or release of soluble target cell-derived factors that specifically modulate autoreactive cells (5–7), reducing the level of direct cell-mediated injury. Alternatively, some cell types have intrinsic resistance to the toxic effects of self-effector molecules that otherwise potently destroy many pathogens (8). Overall, these pathways are best understood in the context of cell-mediated immunity [e.g., induction of anergy or deletion in responding T cells and/or resistance to effector pathways such as perforin, granzyme, and cytokine-mediated killing (9–12)].

In contrast to intrinsic resistance to cell-mediated destruction, intrinsic resistance to humoral immunity poses a distinct challenge.

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Received for publication April 3, 2013. Accepted for publication September 6, 2013.

This work was supported by discretionary funds provided by Emory University and the Puget Sound Blood Center (to J.C.Z.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: DdD, 1,1′dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate; Dil, 1,1′dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DiO, 3,3′-dioctadecyloxycarbocyanine perchlorate; GPA, glycoporphrin A; HOD, hen egg lysosome-OVA–Duffy; K0, knockout; WT, wild-type.

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induced alteration of recognized Ag as a mechanism to decrease surface-bound Abs.

Ag modulation has been difficult to study in vivo, because the target cells typically have ongoing synthesis of the target Ag, resulting in steady state equilibria that complicate analysis of the fate of particular Ag molecules (21, 25). Moreover, as target cells have ongoing division, tracking their numerical destruction over time (or lack thereof) after Ab binding is likewise challenging (21, 25). To circumvent both of these limitations, we have made specific use of a model of Ag modulation using RBCs as cellular targets. Mature RBCs are postmitotic and lack ongoing protein synthesis (26). It is for this reason that transfer of a distinct RBC population into an Ag+ recipient allows examination of specific consequences, including cellular clearance and the potential development of protection against Ab-mediated removal, without the confounding variables of cell division or synthesis of additional protein. In this study, we use a recently described RBC Ag–Ab system using RBCs that express a unique model Ag: the hen egg lysozyme (HEL)–OVA–Duffy (HOD) Ag (27, 28). Using this system, we describe RBCs escaping destruction by Abs through Ag modulation. More importantly, the clearance mechanisms require FcγRs, but not complement. This feature allows a mechanistic focus on Ag modulation and its effects in an isolated FcγR-dependent pathway. We report that in addition to mediated clearance, FcγRs are themselves instrumental in bringing about Ag modulation of Ab targets. Taken together, these results elucidate details of a poorly understood mechanism of cellular alteration in response to Ab binding that may lead to protection against FcγR-mediated effector responses in vivo.

Materials and Methods

Mice

C57BL/6 (B6), FVB, and C3 knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FcγR KO mice (Fcεr1g−) were purchased from Taconic Farms. All mice were used at 8 to 14 wk of age. HOD mice consisted of transgenic animals (FVB background) expressing a fusion protein HEL, Ova, and Duffy b (also known as HOD mice) (27). All breeding (including FcγR KO and C3 KO mice) was performed by the Emory University Department of Animal Resources Husbandry Services, and all procedures were performed according to approved Institutional Animal Care and Use Committee protocols.

Abs and passive immunization

The mouse anti-Fy3 (anti-HOD, MIMA29, IgG2a) and mouse anti-M (anti-GPA, 6A7, IgG1) were a generous gift from Marion Reid and Greg Halverson at the New York Blood Center. Abs were purified by protein G chromatography (Bio X Cell, West Lebanon, NH). The wild-type (WT) C57BL/6 mice were passively immunized with 50 μg 200 μg MIMA29 in PBS or 25 μg 6A7 in PBS, or were given PBS alone by tail-vein injection 2–5 h prior to transfusion.

Fluorescent labeling and transfusion of murine RBCs

HOD transgenic mice, HOD X GPA transgenic mice, and WT FVB mice were anesthetized with isoflurane and exsanguinated by enucleation. Blood collected was washed with PBS and labeled with one of three different lipophilic dyes used to track the survival of these RBCs posttransfusion, as outlined previously (29, 30). Briefly, HOD blood was typically labeled with 1.1′-dioctadecyl-3,3′,3′′-tetramethylindocarbocyanine perchlorate (DiI), and FVB blood was typically labeled with 3,3′-dihexadecyloxacarbocyanine perchlorate (DiD). After labeling, HOD and WT FVB blood cells were mixed at a 1:1 ratio and brought to a 20% hematocrit with LPS-free PBS. After labeling, HOD (Ag+) cells was labeled with a different fluorescent dye, DiD, to enable direct flow cytometric examination of Ag+ cells following adoptive transfer into HOD− (Ag−) recipients, we labeled HOD (Ag+) cells with the fluorescent dye DiI, to enable direct flow cytometric examination of Ag− cells following Ab engagement in vivo (Fig. 1A). To determine whether potential cellular alterations specifically occurred on Ag+ cells within a given recipient, a second population of Ag− (Ag−) cells was labeled with a different fluorescent dye, DiO, to serve as an Ag− control (Fig. 1A).

Adoptive transfer of either DiI- or DiO-labeled RBCs, followed by evaluation of recipient peripheral blood by flow cytometry, demonstrated that single labeling resulted in a distinct and detectable population for either DiO (Fig. 1B) or DiI (Fig. 1C). Moreover, neither DiO nor DiI cells cross-fluoresced on the channel of the other dye. Staining with anti-TER119, an RBC-specific Ag, confirmed that essentially 100% of events were TER119+ after gating on DiI or DiO populations (Fig. 1B, 1C). Finally, DiI-labeled Ag+ RBCs and DiO-labeled Ag− RBCs were mixed prior to adoptive transfer, and peripheral blood of recipients was analyzed. Not only were the DiO and DiI populations easily distinguishable, but the Ag+ HOD RBCs (DiI+) stained positive with an Ab to HOD (anti-Fy3), whereas no staining was observed on WT Ag RBCs (Fig. 1D). Thus, in aggregate, this technique allows the introduction of RBCs into a recipient that has Abs
against a defined Ag, combined with the ability to simultaneously evaluate RBC survival and to visualize and characterize alterations to the targeted Ag. Owing to the nature of RBCs, neither cellular division nor new Ag synthesis is able to confound interpretation of cellular survival or Ag modulation.

In vivo binding of Ab to RBC Ag and the effects on RBC survival

To study the effects of Ab engagement on RBC survival, a mixture of Ag+ (DiO) and Ag- (DiI) RBCs was transferred i.v. into mice that had been passively immunized with a mouse IgG2a mAb against one epitope on the HOD Ag (anti-Fy3). In any given recipient, survival of Ag+ RBCs was normalized to survival of Ag- RBCs, to allow an internal control for any variability in adoptive transfer or bloodletting. Control animals that were not immunized (PBS injected) were used to establish the baseline survival of Ag+ RBCs in the absence of Ab binding. To evaluate binding of anti-Fy3 to Ag+ RBCs, peripheral blood of recipients was stained with anti-Ig and evaluated by flow cytometry. Anti-Ig was nonreactive with either Ag+ or Ag- RBCs in control mice injected with PBS (Fig. 1E). In contrast, Ag+ RBCs in immunized mice were strongly reactive with anti-Ig, whereas no signal was detected on Ag- RBCs (Fig. 1F). Together, these data demonstrate selective in vivo binding of anti-Fy3 to Ag+ RBCs expressing the HOD Ag.

Analysis of RBC survival over time indicated that Ag+ RBCs had a dramatically decreased survival compared with survival in RBCs to allow an internal control for any variability in adoptive transfer or bloodletting. Control animals that were not immunized (PBS injected) were used to establish the baseline survival of Ag+ RBCs in the absence of Ab binding. To evaluate binding of anti-Fy3 to Ag+ RBCs, peripheral blood of recipients was stained with anti-Ig and evaluated by flow cytometry. Anti-Ig was nonreactive with either Ag+ or Ag- RBCs in control mice injected with PBS (Fig. 1E). In contrast, Ag+ RBCs in immunized mice were strongly reactive with anti-Ig, whereas no signal was detected on Ag- RBCs (Fig. 1F). Together, these data demonstrate selective in vivo binding of anti-Fy3 to Ag+ RBCs expressing the HOD Ag.

Analysis of RBC survival over time indicated that Ag+ RBCs had a dramatically decreased survival compared with survival in
Ag⁻ RBCs in mice that received anti-Fy3 but had a normal survival in control animals that received PBS (Fig. 1G). However, clearance kinetics were neither linear nor uniform. Although nearly 40% of Ag⁺ cells cleared within the first 2 h following adoptive transfer, 2 d were required for an additional 40% to clear (Fig. 1G). These clearance kinetics indicate an alteration to the biology regarding RBC clearance after 2 h.

**RBCs surviving despite Ab binding are resistant to clearance**

The existence of a rapid phase of RBC clearance followed by significantly slower clearance raised the hypothesis that a subpopulation of RBCs may resist clearance mechanisms. However, these findings were also compatible with alternative and more trivial hypotheses, in particular, depletion of Ab or saturation of RBC clearance mechanisms. To test these hypotheses, two separate approaches were taken. In the first approach, a two-stage transfer experiment was carried out (see Fig. 2A for diagram). In the first transfer, a mixture of Ag⁺ (DiI) and Ag⁻ (DiO) RBCs was transferred into animals immunized with anti-Fy3 (as above), and the rapid phase of RBC clearance was allowed to occur. At 2 d later, the RBCs that had survived were recovered by exsanguinating recipient mice. The recovered RBCs were then transferred to new mice (second transfer), which had been passively immunized with anti-Fy3. In this way, surviving RBCs were exposed to a fresh dose of Ab and RBC clearance machinery that had not yet consumed Ab-bound RBCs. The above hypotheses predict that if the slowed clearance after 40% removal was due to Ab depletion or saturation of clearance mechanisms, then a new “rapid phase” should be observed during the second transfer. In contrast, if the RBCs have a phenotype resistant to clearance, then in the second transfer, they should have kinetics similar to those of the slow clearance phase.

During the first transfer, and consistent with results in Fig. 1, Ag⁺ RBCs had a rapid clearance phase followed by a slower clearance phase (Fig. 2B). However, only a small and limited rapid clearance phase was observed during the second transfer (Fig. 2C). Rather, the majority of Ag⁺ RBCs cleared at the slower rate, consistent with the second phase of clearance (see Fig. 1). This result was not due to simply transfusing a smaller number of RBCs as a result of the rapid clearance phase eliminating 40% of RBCs, as decreasing the RBC number of Ag⁺ RBCs during the first transfer had the opposite effect (Supplemental Fig. 1). It was not possible in the second transfer to have a control group that had no Ab bound, as the Ab from the first transfer remains bound when put into a second mouse (Supplemental Fig. 1). However, we did control for the possibility that slower clearance was due to just having circulated in a mouse (See Fig. 2A, group 2), which did not prevent the rapid clearance phase in the second transfer but instead resulted in enhanced clearance (Fig. 2D).

Although these results suggested that Ag⁺ cells may acquire resistance to Ab-mediated removal, this approach did not specifically determine whether immunized recipients retain the capacity to clear Ag⁺ cells following the development of reduced rates of clearance. To test this, Ag⁺ cells were transferred into immunized recipients, followed by a second transfer of Ag⁺ cells into the same recipients 2 d later. As a control, immunized recipients not previously exposed to Ag⁺ cells were transferred with Ag⁺ cells in parallel (Fig. 3A). Ag⁺ cells experienced similar levels of clearance in immunized recipients that previously received Ag⁺ cells as immunized recipients not previously exposed to Ag⁺ cells, strongly suggesting that immunized recipients retain the ability to remove Ag⁺ cells following the development of reduced rates of clearance (Fig. 3B, 3C). As a whole, our interpretation of these data is that Ab-bound HOD Ag⁺ RBCs that survive the rapid clearance phase after initial transfer are resistant to subsequent clearance.

**Clearance of HOD RBCs by anti-Fy3 requires FcγRs, but not C3**

To further investigate mechanisms of clearance resistance, it was necessary to establish the fundamental mechanisms of clearance itself. The two main pathways involved in clearance of Ab-coated...
RBCs in vivo involve either FcγRs or complement. However, alternative pathways have also been described that do not require either FcγRs or complement (28). Thus, to evaluate clearance mechanisms of anti-Fy3 and HOD, transfer experiments were carried out in mice with targeted deletions of either the C3 component of complement or the common γ-chain of FcγRs.

C3 is the main complement opsonin that causes RBC consumption and is also instrumental to downstream formation of the membrane attack complex. Clearance in C3 KO mice had a kinetics and magnitude similar to that observed in WT recipients (Fig. 4A). To test whether C3 is deposited on Ag+ RBCs after anti-Fy3 binding, peripheral blood samples were obtained at 2 h post-transfusion from WT recipients and were stained with anti-C3. Anti-C3 reactivity was detected in the presence of anti-Fy3 in a population of Ag+ (DiI) RBCs (Fig. 4B). This C3 deposition required the presence of the target Ag, as no C3 was detected on Ag- RBCs (DiO) (Fig. 4B). The deposition of C3 on HOD RBCs was not a spontaneous event or a function of the nonclassical pathway, as no C3 was detected on either Ag+ or Ag- RBCs in control mice that received PBS (Fig. 4B). Thus, deposition of C3 in this system requires that Ag and Ab be simultaneously present. Representative flow plots during clearance in the C3 KO mice are shown and, as predicted owing to the targeted deletion of C3, no anti-C3 reactivity is observed in either group (Fig. 4C). Together, these data indicate that C3 is indeed deposited on the surface of some HOD RBCs after binding of anti-Fy3; however, the functional effect of C3 binding is unclear with regard to clearance, as clearance curves are normal in the C3 KO animal. Thus, these data do not rule out the involvement of C3 but do demonstrate that C3 is not required for clearance in this system.

Although C3 engagement is thought to be the primary complement effector pathway whereby Abs initiate complement, several C3-independent pathways of complement activation have been described (32), suggesting that downstream targets of com-

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**FIGURE 4.** Clearance of HOD RBCs by anti-Fy3 requires FcγRs, but not C3. (A) Clearance kinetics of Ag+ RBCs in C3 KO mice immunized with anti-Fy3 or saline. (B) Flow cytometric examination of Ag+ cells (solid black line) or Ag- cells (gray) for surface C3 binding following transfer into WT recipients. (C) Representative DiI × DiO plots for calculating RBC survival and flow cytometric examination of Ag+ cells (solid black line) or Ag- cells (gray) for surface C3 binding following transfer into C3 KO recipients. (D) Clearance kinetics of Ag+ RBCs in FcγR KO mice immunized with anti-Fy3 or saline. Representative flow plots are reflective of three independent experiments. Each experiment represents three to five mice per group at each time point analyzed.
plement activation may be involved in cellular clearance. To test this, we next transferred Ag⁺ cells into immunized FVB recipients, which are deficient in the C5 component of complement required for initiation of the membrane attack complex (33). Immune FVB recipients displayed a similar ability to clear Ag⁺ cells (Supplemental Fig. 2), strongly suggesting that Ag⁺ cell clearance occurs independent of the downstream complement effector molecules. Thus, these data do not rule out the involvement of C3 or C5 but do demonstrate that C3 and C5 are not individually required for clearance in this system.

To determine the role of FcγRs in this process, the same experiment was carried out in mice with a targeted deletion of the R mice that were immunized with anti-Fy3, whereas FcγRII and FcγRIV (16). No clearance of Ag⁺ cells was observed following transfer into immunized FcγR KO recipients (Fig. 4D), indicating that FcγRs are required for RBC clearance in this system. Because essentially no clearance was observed in the FcγR KO mice, this also suggests that complement is not sufficient to induce RBC clearance. Together, these data indicate that FcγRs are required for clearance of HOD RBCs by anti-Fy3 and that C3 is neither required nor sufficient for clearance.

RBCs resistant to clearance undergo Ag modulation of surface HOD that correlates with resistance to clearance

To further characterize Ag–Ab interactions on the RBCs, we directly examined Ag⁺ cells for Ab engagement at various points following adoptive transfer into immunized recipients. Peripheral blood was collected at the indicated time points, stained with anti-lg, and analyzed by flow cytometry (Fig. 5A). Although the level of bound Ab remained high during the initial rapid phase of clearance, the amount of detectable Ab on Ag⁺ cells progressively decreased over time (Fig. 5A). The staining was specific for the presence of anti-Fy3, as no signal was detected on Ag⁺ RBCs (Fig. 5A) or on either Ag⁺ or Ag⁻ RBCs in control mice that received PBS (Fig. 5A and Supplemental Fig. 3).

As immunization of recipients reflected passive transfer of Ab, it remained possible that reduced Ab engagement simply reflected rapid consumption of passively administered Ab. However, staining of recovered RBCs with anti-Fy3 itself, followed by anti-lg, failed to increase the signal, indicating that the available anti-Fy3 binding sites were saturated (Fig. 5B). In contrast to the observed decrease in Ag seen on Ag⁺ RBCs in anti-Fy3–immunized mice, Ag⁺ RBCs recovered after transfer into PBS–treated mice retained full staining (Fig. 5B, Supplemental Fig. 3). Apparent loss of detectable Ab did not appear to reflect wholesale loss of the plasma membrane, as cells experiencing Ag modulation isolated from immunized recipients displayed forward scatter profiles very similar to those of Ag⁺ cells transferred into nonimmunized controls (Fig. 5C, 5D). Together, these data indicate that the amount of detectable Ag on the transferred Ag⁺ RBCs decreases as a function of being transferred into an animal with circulating anti-Fy3, and establishes the phenomenon of Ag modulation in this system.

Effect of Ab density on RBC Ag decrease and clearance

The above data indicate that the decrease in levels of Ab engagement on Ag⁺ cells paralleled the decreased kinetics of RBC clearance and the generation of RBC resistance to clearance (see Figs. 1G, 2). These observations led us to hypothesize that alterations in surface RBC Ag, and thus decreased Ag engagement, was involved with decreased RBC clearance. However, it is important to note that although Ag modulation decreases detectable Ag, Ag-bound Ab remains readily detectable. Thus, for Ag modulation to have an effect on RBC clearance, there would have to be a density dependence of FcγR ligation. To test this hypothesis, we incubated cells with varying levels of Ab or no Ab in vitro prior to adoptive transfer in an effort to recapitulate the potential impact of reduced Ab levels on Ag⁺ cell removal (Fig. 6A). Incubation of Ag⁺ cells with Ab in vitro enabled isolation of RBCs with Ag saturation [hi], an Ab intermediate population [int], or cells with no Ab [0] prior to transfer (Fig. 6B, Supplemental Fig. 3). Each population of RBCs was then transferred into WT mice (no passive Ab into the mice), and mice were bled at the indicated time. The amount of surface IgG was determined by staining with anti-Ig, whereas the amount of Fy3 Ag was determined by staining with anti-Fy3 followed by anti-lg.

Compared with control HOD RBCs that were transferred without preincubation with Ab (Fig. 6C, top row), transfer of Ab-saturated cells [hi] displayed significant decrease in bound Ab over time (Fig. 6C, bottom row). Likewise, staining with anti-Fy3 followed by anti-lg demonstrated decreased detectable Ag in [hi] RBCs (Fig. 6D, bottom row). This observed pattern in [hi] RBCs was similar to what was observed following adoptive transfer of Ag⁺ cells into immunized recipients (see Fig. 5). In contrast, [int] cells failed to display a similar decrease in surface Ab from pre-transfer levels (Fig. 6C, middle row). Moreover, staining with anti-Fy3 and anti-lg increased the signal on [int] cells to a level similar to that in control HOD RBCs that were transferred without preincubation with Ab (Fig. 6D, middle and top rows; Supplemental Fig. 3). Thus high levels of Ab, but not intermediate levels, result in Ag modulation over time.

To test the effect of [0], [int], and [hi] levels of surface RBC Ab on RBC survival after transfer, circulating Ag⁺ RBCs were enumerated by the same Dil/DIO techniques used above. Whereas [hi] levels of Ab caused rapid clearance, [int] levels of Ab caused substantially slower clearance (Fig. 6E). The [hi] and [int] Ab incubations were titrated to mimic Ab levels on Ag⁺ RBCs prior to transfer and after the rapid clearance phase, respectively. Thus, these findings are consistent with Ag modulation, resulting in a sufficient decrease in surface Ab to render RBCs resistant to clearance. Together, these data indicate that both clearance and Ag modulation are a function of the amount of RBC surface-bound Ab.

FcγRs, but not C3, is required for decrease in detectable Ag

Although C3 was neither required nor necessary for clearance of transferred HOD RBCs, this does not exclude its possible role in Ag modulation. Indeed, Taylor et al. (34) have reported that C3 deposition on Ig itself can prevent binding of secondary Abs through direct blocking or steric hindrance. To test this hypothesis, Ag modulation was evaluated after transfer into C3 KO mice. Transfer of Ag⁺ cells into immunized C3 KO mice resulted in a decrease in detectable Ag similar to that in WT recipients (Fig. 7A, 7B). Similar results were obtained following transfer of Ag⁺ cells into C5-deficient FVB immunized recipients (Supplemental Fig. 4). These data indicate that C3 and C5 are not required for Ag modulation in this system, and we thus reject the hypothesis that the mechanism of Ag decrease is masking by C3 or C5 deposition.

To test the requirement for FcγR for Ag modulation, a mixture of Ag⁺ and Ag⁻ cells was transferred into FcγR KO immunized recipients. In the absence of FcγRs, Ab persisted on cells unaltered over extended periods (Fig. 7C). As above, RBCs did not clear in FcγR mice that were immunized with anti-Fy3, whereas normal clearance occurred in WT animals (Fig. 7D–F). These data indicate that FcγRs are required for the process of Ag modulation.

Decrease in detectable Ag extends intra- but not intermolecularly

To determine whether Ag modulation was specific to the epitope recognized by anti-Fy3, we compared levels of Fy3 with an epitope
on the opposite end of HOD (Fy6; see Fig. 8A). Ag+ cells were isolated at various time points following transfer and were incubated with an anti-Fy6 Ab, followed by detection of total Ab bound to cells, similar to the method used to determine whether alterations in the Fy3 target epitope occurred over time. Although the Fy6 epitope remained detectable, with unaltered levels of Ag on cells transferred into nonimmunized recipients, the Fy6 epitope experienced a decrease similar to that observed for the Fy3 epitope over time in immunized mice (Fig. 8B–D). These data indicate that a separate epitope on the same molecule, and not recognized by anti-Fy3, undergoes a simultaneous and similar decrease in detectability.

To test whether Ag modulation was generalized to epitopes on separate molecules, two approaches were taken. In the first approach, we tested the expression of Ter119, an RBC-specific Ag unrelated to HOD. Ter119 RBC Ag expression remained unchanged over the same time and under the same conditions (Fig. 8E). In the second approach, we crossed HOD transgenics by transgenics expressing the human glycophorin A (GPA) Ag, a human Ag similar to the mouse Ter119 Ag, on RBCs and transferred these double transgenic mice into immunized and nonimmunized recipients.
positive cells into nonimmunized, anti-Fy3–immunized, or anti-
GPA–immunized recipients. Although nonimmunized recipients
failed to clear double Ag⁺ cells, anti-Fy3–immunized and anti-
GPA–immunized recipients each effectively cleared double positive
cells (Fig. 9A), consistent with previous results and those of this
study (35). Examination of bound Ab and detectable Ag demon-
strated that Ab could be detected only on double Ag⁺ cells following
transfer into immunized recipients and that double positive RBCs
experienced modulation of HOD Ag, but not GPA Ag, following
transfer into anti-Fy3–immunized recipients (Fig. 9B). Conversely,
transfer of double Ag⁺ cells into anti-GPA–immunized recipients
likewise resulted in Ag modulation of the GPA Ag, whereas the
HOD Ag remained unaltered (Fig. 9B). Together, these data indicate
that a decrease in detectable Ag appears to reflect specific modu-
lation of the target Ag following Ab engagement.

**Discussion**

Innate immunity and adaptive immunity have evolved multiple
effectector pathways to protect the host from pathological microbes.
Abs represent one such pathway and have the capacity to destroy
the targets they bind through both fixation of complement and
ligation of FcγRs. However, the presence of such Abs necessitates
an intrinsic risk of damaging self-tissues. As an evolutionary re-
response to this damage, multiple pathways have been described

**FIGURE 6.** Cells with intermediate levels of Ab exhibit reduced sensitivity to clearance. (A) Schematic representation of the experimental design. (B) Distinct populations of Ag⁺high or Ag⁺intermediate cells transferred into nonimmunized recipients. (C) Examination of Ab bound to transferred cells at the indicated time points on Ag⁺ cells (solid black line) or Ag⁻ cells (gray). (D) Examination of levels of detectable Ag on Ag⁺ cells (solid black line) or Ag⁻ cells (gray) at the indicated time points. (E) Clearance kinetics of RBCs pretreated with [0], [int], or [hi] concentrations of anti-Fy3, followed by transfer into WT recipients. Representative flow plots are reflective of three independent experiments. Each experiment represents three to five mice per group at each time point analyzed.
by which self-tissues can inactivate complement, thus mitigating damage from autoantibodies or alloantibodies. In contrast, little has been described regarding FcγR-mediated pathways and the extent to which protection pathways have evolved. One pathway that has been put forward is referred to in this article as “Ag modulation,” a process by which target Ags change so as to decrease Ab binding and thus limit FcγR ligation. Detailed analysis of the effects of Ag modulation requires a system in which FcγRs are isolated as an Ab effector pathway (i.e., complement does not play a role). Moreover, one must control issues of cellular proliferation and Ag resynthesis by target cells during any ongoing Ag modulation. In this article, we describe just such a system by taking advantage of RBCs, which neither proliferate nor synthesize protein and are cleared by an FcγR dependent/complement-independent pathway.

The data presented generate novel understanding of Ag modulation at several different points. First, the presence of both anti-Fy3 and FcγRs is required for both RBC clearance and Ag modulation. Hence, the biology and the multiple effects of Ab-induced FcγR ligation overlap. One trivial explanation that might come from these observations would be that RBCs with a baseline level of higher Ag expression are cleared, whereas those with a baseline lower Ag expression are not. Thus, what appears to be ongoing Ag modulation on RBCs actually just represents selective removal of a population with high Ag expression. However, we reject this interpretation as mathematically inconsistent with staining of RBCs at baseline. After 2 h, 50% of the Ag+ RBCs are still circulating and have a 5-fold decrease in surface Ag. However, prior to transfer into immunized animals, only a very small percentage of RBCs have lower baseline levels of Ag on the surface (see Fig. 1). Accordingly, our interpretation is that surviving RBCs started with high levels of detectable Ag and underwent an Ag modulation process that substantially decreased detectable Ag.

In the above context, the simultaneous requirement for FcγR to carry out both clearance and Ag modulation is a unique and...
novel finding. This indicates an FcγR-dependent biology that results in the modulation of presynthesized Ag on the RBC surface. The precise details of the mechanism by which this occurs are not clear at all levels; however, we can make some distinct conclusions based upon the current data. First, according to published literature, C3 that deposits on cell-bound Ab can modify it such that anti-Ig no longer recognizes the Ab (34, 36, 37). Given that C3 deposition was detected after anti-Fy3 bound to Ag+ RBCs (see Fig. 3), and given the known phenomenon, this seemed a likely hypothesis. However, we reject this hypothesis based upon the fact that Ag modulation occurs unabated in C3 KO mice. Similar results were observed following transfer into immunized FVB C5-deficient recipients (33). This observation does not address the more general issue of any modification of anti-Fy3 (other than involving C3 or C5) that may render it undetectable by anti-Ig. However, the observation that both Fy6 and Fy3 undergo Ag modulation after exposure to anti-Fy3 provides additional evidence that simple modification of the anti-Fy3 is not a likely mechanism. Together, these data indicate that the mechanism of Ag modulation is not modification of the Ag-bound Ab, and likely represents alteration to the Ag itself.

The exact fate of the HOD Ag, after binding of anti-Fy3, is not clear; indeed, possibilities include complete removal from the cellular membrane, partial removal of select epitopes, or protein modification so as to render the Ag undetectable by Ab. The modulation of Fy6 by anti-Fy3 rejects the hypothesis that anti-Fy3 is causing an epitope-selective destruction of the protein. Moreover, reduction of the protein and chemical denaturation seem unlikely mechanisms, as both anti-Fy3 and anti-Fy6 recognize linear peptide sequences. In addition, the selective modulation of the Fy3 Ag following transfusion of HOD X GPA RBCs also suggests that anti-Fy3 engagement results in specific alterations to the target Ag. Although complete removal of a transmembrane protein from the RBC surface, without damaging the RBC, may seem unlikely, at least one human case of Ag modulation during autoimmune hemolytic anemia provided substantial evidence that the Ag was removed from the RBC (38). This removal would not necessarily require pulling the protein out of the membrane, which seems thermodynamically unlikely. Rather, it may involve trogocytosis of aggregated Ag complexes, which has been observed in the context of Ag modulation of CD20 by chronic lymphocytic leukemia in response to mAb immunotherapy (i.e., rituximab) (25). As some cases of autoimmune hemolytic anemia, in which changes in cell size may be detectable, reflect Ab engagement of high-density Ags (39), the lack of detectable alterations in cell size in the present system with a corresponding reduction in the amount of membrane removed. As a result, subtle changes in Ag levels, as observed following Ag modulation, may not produce enough loss of membrane to be detected with the tools used in this study. As a result, we propose a model by which Ab binding Ag and ligating FcγR results in removal of the Ag from the membrane.

The process of Ag modulation has been observed in numerous settings, and strong human data indicate that Ag modulation occurs by several different mechanisms, depending upon the nature of the Ab, the Ag, and the target cell. Very much as in humans, the same diversity of mechanisms appears to be present in mice. We have previously reported that Ag modulation of murine HEL on RBCs requires the simultaneous binding of two separate Abs that bind to distinct epitopes. However, Abs that engage HEL in this system fail to induce RBC clearance, making it difficult to determine whether Ag modulation can protect cells from Abs capable of inducing RBC removal. However, such appears not to be the case in the current

![FIGURE 8](http://www.jimmunol.org/)

Anti-Fy3 induces coincident Ag modulation of both Fy3 and a third party Ag, Fy6. (A) Schematic of the HOD Ag. (B and C) Examination of detectable Ag for Fy6 (B) or Fy3 (C) on Ag+ cells (solid black line) or Ag- cells (gray) at the time points indicated following transfer into nonimmunized or immunized recipients. (D) Examination of Ab on Ag+ cells (solid black line) or Ag- cells (gray) at the time points indicated following transfer into nonimmunized or immunized recipients. (E) Examination of cell surface Ter119 staining on Ag+ cells (solid black line) or Ag- cells (gray) 5 d following transfer into immunized or nonimmunized recipients, as indicated. Ag+ cells (black dotted line) or Ag- cells (light gray) stained with isotype control are also shown. Representative flow plots are reflective of three independent experiments. Each experiment represents three to five mice per group at each time point analyzed.
setting, as a monoclonal anti-Fy3 resulted in Ag modulation and clearance. Similarly, the ability of anti-GPA to induce Ag modulation demonstrates that Ag modulation is not limited to anti-Fy3. The unique biphasic clearance pattern induced by anti-GPA is consistent with previous studies (35). Given the potential involvement of complement in anti-GPA–mediated clearance (40), complement receptors may initially trap RBCs following Ab-mediated complement deposition and then release some RBCs following subsequent complement degradation, as suggested by previous studies examining the consequences of complement deposition on RBCs (36, 41). Although additional studies are needed to further understand the mechanisms of anti-GPA–induced clearance and Ag modulation, these results suggest that alterations in Ag following Ab engagement may reflect a general mechanism of cellular protection from Ab-mediated removal. Thus, although the current findings cannot be used to draw generalized conclusions regarding Ag modulation, Ag modulation appears to reflect a general phenomenological descriptor that captures several different processes with a common outcome. However, to the best of our knowledge, the current report represents the first description of a new pathway of Ag modulation, in which FcγRs are required both for clearance and for the process of Ag modulation itself.

Although Ag modulation and clearance appear to use the same FcγR system, whether the same FcγRs mediate clearance and Ag modulation remains unknown. Previous studies suggest that IgG2a Abs preferentially engage FcγRII, whereas IgG1 and IgG3 prefer to bind FcγRIII and FcγRI, respectively (42, 43). As FcγR KO recipients were used in the current study, it remains possible that the IgG2a anti-Fy3 used in this study preferentially triggers FcγRIIs following engagement of Ag+ cells in vivo. In contrast, class switch variants of anti-Fy3 may preferentially engage alternative FcγRs. As it remains possible that the FcγRs responsible for clearance may be different from those that induce Ag modulation, manipulation of Ab subclass may also enable preferential engagement of clearance or Ag modulation pathways. Similarly, although many different cells express FcγRs and therefore may be involved in Ag+ cellular clearance or Ag modulation (43), previous studies suggest that various subsets of macrophages may be involved in RBC clearance (44), indicating that these cells may be uniquely positioned to mediate Ag+ cell removal following Ab engagement. However, whether these same cells engage both clearance and modulation pathways remains unknown, providing another possible target that may facilitate differential induction of Ag modulation versus clearance following Ab engagement in vivo. Future studies will consider these possibilities.

In the current studies, using retransfer experiments, we demonstrated that there is a subset of RBCs resistant to clearance by an FcγR-dependent process (see Fig. 2) and also showed that the kinetics of Ag modulation correlated to this clearance. Technical limitations prevented us from unequivocally testing the interpretation that Ag modulation is responsible for resistance (i.e., to treat RBCs such that Ag modulation does not occur and determine whether all of the RBCs then clear). However, our Ab titration studies indicate that the level of Ab bound after Ag modulation results in decreased clearance (see Fig. 6). These data strongly suggest that, indeed, Ag modulation is capable of rendering cells resistant to clearance by FcγR-dependent pathways.

The high level of Ag on Ag+ cells prior to transfer suggests that all cells should be equally sensitive to clearance following engagement of Ab in vivo. However, only 40% of Ag+ cells rapidly clear following transfer, with the remaining cells adopting a much slower rate of clearance, consistent with the possible development of cellular resistance to Ab-mediated removal. As the FcγR system appears to be involved in both clearance and Ag modulation, the failure of recipients to clear 100% of cells following transfer likely reflects a dynamic process of simultaneous Ag modulation.

![FIGURE 9](http://www.jimmunol.org)
and clearance; cells that undergo Ag modulation escape the initial wave of clearance, whereas those that fail to rapidly undergo Ag modulation remain sensitive to clearance pathways and are removed from the circulation. However, the factors that dictate which cells will undergo Ag modulation versus clearance remain unknown and will be the subject of future studies.

As many autoantibodies, alloantibodies, and Ab-based therapeutics engage FcγR (43, 45, 46), the present results not only provide significant insight into potential regulatory pathways responsible for modulating FcγR effector function but will likely provide a unique tool to facilitate additional insight into factors that dictate the consequence of FcγR engagement in vivo. For example, although reduced Ab effector function following Ab engagement would likely modulate the outcome of cell survival favorably in settings of autoimmunity or allograft immunization, similar alterations would likely reduce the therapeutic efficacy of Ab-based therapeutics (25). As a result, a greater understanding of the mechanisms and factors responsible for regulating Ab-induced Ag loss have the potential to facilitate the development of novel therapeutics aimed at treating individuals with Ab-mediated disease while also enabling the enhancement of Ab-based therapeutics.

Acknowledgments

We thank Krystal Hudson, Kate Henry, and Geetha Mylvaganam for helpful discussions.

Disclosures

J.C.Z. has sponsored research agreements with Immucor and Terumo, neither of which is related to the work in this study.

References


SUPPLEMENTAL FIGURE LEGENDS:

Supplemental Figure 1 RBC clearance and antibody engagement after transfer Ag+ cells into immunized recipients. (A) Quantification of Ag+ cell clearance 2 hours following transfer of 500 µl containing 20% Hct (RBC) or 500 µl containing 0.2% Hct (RBC lo) into non-immunized or immunized WT recipients (B) Flow cytometric examination of Ag+ cells (red line) or Ag- cells (blue line) for bound antibody 2 days following transfer into immunized recipients and prior to transfer into non-immunized or newly immunized recipients. Representative flow plots are reflective of three independent experiments. Each experiment represents 3 to 5 mice per group.

Supplemental Figure 2 RBC clearance following transfer into non-immunized or immunized FVB recipients. Clearance kinetics of Ag+ RBCs in non-immunized or immunized FVB recipients at various time points as indicated. Data are representative of at least three independent experiments and each experiment represents 3 to 5 mice per group at each time point analyzed.

Supplemental Figure 3 Antigen positive cells undergo antigen modulation and cells with intermediate levels of antibody exhibit reduced sensitivity to clearance (Data displayed as stacked histograms. See figures 5A,B and 6B-D for comparison). (A) Flow cytometric examination of Ag positive cells or Ag negative cells for bound antibody following transfer into immunized or non-immunized recipients at the times indicated. (B) Flow cytometric examination of Ag positive cells or Ag negative
cells for detectable antigen following transfer into immunized or non-immunized recipients at the time points indicated. (C) Distinct populations of antigen high [hi] or antigen intermediate [int] cells transferred into non-immunized recipients. (D) Examination of antibody bound or levels of detectable antigen on transferred cells with [0], [int], or [hi] concentrations of anti-Fy3 at the indicated timepoints on Ag positive cells or Ag negative cells. Representative flow plots are reflective of three independent experiments. Each experiment represents 3 to 5 mice per group at each time point analyzed.

**Supplemental Figure 4** Antigen modulation occurs following transfer of Ag positive cells into immunized FVB recipients. Examination of bound antibody or detectable antigen 2 days following transfer of Ag+ cells (solid black line) or Ag- cells (gray) into non-immunized or immunized FVB recipients. Representative flow plots are reflective of three independent experiments. Each experiment represents 3 to 5 mice per group at each time point analyzed.
Supplemental Figure 1

A

B
Supplemental Figure 2

Clearance in FVB recipients

% of cells remaining vs. Time (hrs)

- PBS
- α-Fy3
Supplemental Figure 4