

## SSI 50 YEARS ANNIVERSARY ARTICLE

## SPECIAL REVIEW

# IgG-mediated suppression of antibody responses: Hiding or snatching epitopes?

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**Abstract**

Antibodies forming a complex with antigen in vivo can dramatically change the antibody response to this antigen. In some situations, the response will be a 100-fold stronger than in animals immunized with antigen alone, and in other situations, the response will be completely suppressed. IgG is known to suppress the antibody response, for example to erythrocytes, and this is used clinically in Rhesus prophylaxis. The mechanism behind IgG-mediated immune suppression is still not understood. Here, we will review studies performed in experimental animal models and discuss the various hypotheses put forward to explain the profound suppressive effect of IgG. We conclude that an exclusive role for negative regulation of B cells through FcγRIIB, increased clearance of erythrocytes from the circulation or complement-mediated lysis is unlikely. Epitope masking, where IgG hides the epitope from B cells, or trogocytosis, where IgG removes the epitope from the erythrocyte, is compatible with many observations. These two mechanisms are not mutually exclusive. Moreover, it cannot be ruled out that clearance, in combination with other mechanisms, plays a role.

## 1 | INTRODUCTION

Antibodies, passively administered to animals or humans, can have profound effects on the immune response to their specific antigen. When preformed antibodies are co-administered with the antigen they recognize, antibody responses can be enhanced more than a 100-fold or suppressed more than 99% as compared to responses in animals immunized with antigen alone (reviewed in Refs1-4). The earliest studies on how antibodies regulate antibody responses were performed

in relation to serum therapy against diphtheria in the end of the 19th century. It was of utmost importance to produce high titre anti-diphtheria toxin to use for treating diphtheria patients. Emil von Behring and coworkers found that immunizing animals with mixtures of antiserum and diphtheria toxin, instead of toxin alone, resulted in production of high titre sera while the toxic effects could be prevented.<sup>5,6</sup> This early observation illustrates the balance between the ability of antibodies to upregulate a response to a protein, for example diphtheria toxin, while at the same time neutralizing its effects. Scandinavian immunologists showed an early interest in antibody feedback regulation with Göran Möller and Hans Wigzell studying the suppressive effects on responses

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to sheep red blood cells (SRBC) in the 1960s.<sup>7,8</sup> It soon became clear that the suppressive effect in SRBC-specific sera was found in the 7S fraction (IgG) and the enhancing effect in the 19S fraction (IgM).<sup>9</sup>

In the following decades, using monoclonal antibodies and gene-targeted mice, the mechanisms behind the enhancing effects of different antibody isotypes were studied in detail. We now know that IgM enhances via the complement system,<sup>10,11</sup> primarily by increasing the amount of antigen localized on follicular dendritic cells.<sup>12,13</sup> IgE enhances antibody and T-cell responses to small proteins by forming an immune complex which binds to the low-affinity receptor for IgE (CD23) on circulating B cells.<sup>14</sup> These B cells have access to the splenic B-cell follicles and substantial amounts of antigen can be detected there 30 minutes after immunization with IgE and antigen.<sup>14,15</sup> The antigen is subsequently presented to CD4<sup>+</sup> T helper cells which become activated and proliferate.<sup>15-17</sup> Presumably, their efficient interaction with cognate B cells leads to the enhanced antibody responses first observed.<sup>18,19</sup> The cells presenting IgE-antigen to T helper cells are dendritic cells<sup>15,20</sup> and not B cells which were initially assumed. Murine dendritic cells do not express CD23<sup>15</sup> and therefore CD23<sup>+</sup> B cells must somehow deliver IgE antigen to them, but exactly how this transfer takes place is not yet understood.

IgG antibodies, either passively administered or actively produced, can suppress the antibody response against the antigen they recognize. In a clinical setting, maternal antibodies inhibit infant antibody responses against many vaccines, such as tetanus, diphtheria, pertussis and hepatitis A (reviewed in Ref21). The ability of passively administered IgG to suppress antibody responses to erythrocytes has been used successfully in the clinic. Administration of IgG anti-Rhesus (Rh)D to RhD-negative women, at risk of becoming immunized against RhD-positive foetal erythrocytes following transplacental haemorrhage, has proven very efficient in preventing haemolytic disease of the foetus and newborn.<sup>22,23</sup> In animals, IgG can suppress responses to proteins administered in adjuvants<sup>24</sup> or in physiological salt solutions,<sup>25,26</sup> as well as to viruses.<sup>27,28</sup> However, the vast majority of studies of IgG-mediated immune suppression have been performed using erythrocytes as antigens and this will be discussed in detail below.

In addition to suppressing antibody responses to, for example, erythrocytes, IgG antibodies of all subclasses can enhance antibody- and T helper cell responses to proteins.<sup>25,29,30</sup> In analogy with IgM, murine IgG3 operates via complement,<sup>31</sup> and the mechanism seems to be that marginal zone B cells, which express complement receptors 1 and 2 (CR1/CR2), transport IgG3-antigen-complement complexes to follicular dendritic cells.<sup>32,33</sup> IgG1, IgG2a and IgG2b are dependent on activating FcγRs for their enhancing effects,<sup>29,30,34,35</sup> and most likely operate by increasing uptake of IgG-antigen by FcγR<sup>+</sup> dendritic cells

for processing and presentation of antigenic peptides to T helper cells.<sup>35</sup>

The focus of this review will be on the ability of IgG antibodies to suppress antibody responses *in vivo*, primarily in mice. The experimental findings will first be presented and subsequently discussed in relation to the major hypotheses proposed to explain the phenomenon:

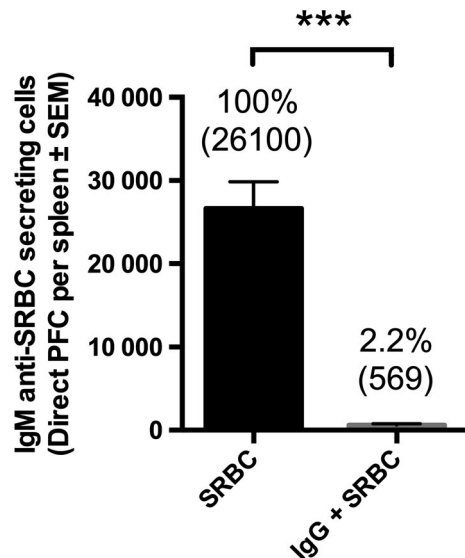
1. Central inhibition of B-cell activation through co-cross-linking of the B-cell receptor (BCR) with the negatively regulating FcγRIIB by IgG-antigen complexes.
2. Complement-mediated lysis of red blood cells.
3. Elimination/clearance of IgG-antigen complexes before they can stimulate an immune response.
4. Masking of epitopes on the antigen, preventing B cells from binding and becoming activated.
5. Trophocytosis/modulation leading to loss of IgG-bound epitopes, preventing B cells from binding and becoming activated.

## 2 | WHICH TYPES OF RESPONSES ARE SUPPRESSED BY IgG?

IgG can suppress antibody responses to proteins<sup>25,26</sup> and viruses,<sup>27,28</sup> but most of our knowledge about the mechanism behind suppression comes from studies using erythrocytes. SRBC or SRBC conjugated to haptens such as trinitrophenyl (TNP) or 4-hydroxy-3-nitrophenylacetyl (NP) has frequently been used.<sup>7,9,36-42</sup> More recently, murine transgenic erythrocytes expressing the entire human KEL glycoprotein (KEL-RBC),<sup>43,44</sup> hen egg lysozyme in sequence with ovalbumin (OVA) and the complete human Duffy<sup>b</sup> transmembrane protein (HOD-RBC),<sup>45-49</sup> or both HOD and KEL antigens<sup>50</sup> were used as antigen.

Many studies of IgG-mediated suppression in mice were performed with Jerne's direct haemolytic plaque forming cell assay, detecting single IgM anti-SRBC-producing cells.<sup>51</sup> With this sensitive method, it was shown that 90%-99% of the IgM responses could be suppressed<sup>9,36-38,52,53</sup> (Figure 1). More recently, splenic germinal centre B cells as well as IgG-producing long-lived plasma cells in the bone-marrow were shown to be suppressed.<sup>40</sup> Also primary serum IgM and IgG responses to xenogeneic<sup>39-41,54</sup> as well as to allogeneic<sup>43-50</sup> erythrocytes are suppressed by IgG.

IgG-mediated suppression is dose dependent: high doses of IgG suppress better than low doses and responses to high doses of antigen are more difficult to suppress than responses to low doses.<sup>9,37,38</sup> Possibly, this explains why secondary antibody responses appear to be more difficult to inhibit than primary responses.<sup>52,55,56</sup> In mice in which IgG suppressed the primary responses, memory/recall responses after boost with antigen alone were also suppressed but not always as



**FIGURE 1** An example of IgG-mediated immune suppression. BALB/c mice were immunized intravenously with 30  $\mu$ g of IgG anti-SRBC, isolated from hyperimmune mouse serum, followed within one hour by  $5 \times 10^7$  SRBC. Controls (black bar) received  $5 \times 10^7$  SRBC alone. Five days later, the number of spleen cells producing IgM anti-SRBC was assayed in Jerne's direct haemolytic plaque forming cell assay. In this example, IgG suppressed 97.8% of the IgM response. \*\*\* $P < .001$

efficiently as the primary response.<sup>27,55-57</sup> In a recent study, the same relative suppression of primary and recall responses was observed in mice primed with IgG anti-SRBC and SRBC and boosted with SRBC 70 days later.<sup>40</sup> This implies that the induction of memory cells and plasma cells was equally well suppressed during priming.

IgG only marginally, if at all, suppresses T helper cell responses in mice in which the antibody responses to SRBC are efficiently suppressed.<sup>38,39,54</sup> In line with this, virus-specific antibodies inhibit the antibody- but not the T-cell responses against viruses in mice<sup>28,58</sup> and humans.<sup>59</sup>

In summary, IgG suppresses both primary and secondary antibody responses and induction of immunological memory, but does not significantly suppress induction of T helper cells.

### 3 | WHAT TYPES OF ANTIBODIES CAN SUPPRESS?

Studies using monoclonal IgG antibodies have shown that all murine subclasses are able to suppress.<sup>36-38,52,60</sup> Notably, this includes IgG3<sup>36,37</sup> which does not bind to the inhibitory Fc $\gamma$ RIIB.<sup>61</sup>

Although IgM usually enhances antibody responses to erythrocytes, suppression has occasionally been demonstrated.<sup>7,36,62</sup> In the latter study,<sup>62</sup> IgM had a dual effect and enhanced when administered before SRBC, but suppressed

when given 2-48 hours after the antigen. This observation is compatible with the finding that IgM enhances by increasing the antigen concentration on follicular dendritic cells.<sup>12,13</sup>

As mentioned in the Introduction, IgE administered with small proteins can enhance antibody responses via CD23. However, monoclonal IgE anti-TNP administered with SRBC-TNP, which is a large antigen, suppressed the antibody response equally well as did monoclonal IgG anti-TNP.<sup>38,63</sup> IgE binds to the low-affinity receptor Fc $\epsilon$ RII (CD23), the high affinity Fc $\epsilon$ RI as well as to some Fc receptors for IgG (Fc $\gamma$ RIIB, Fc $\gamma$ RIII and Fc $\gamma$ RIV).<sup>64-66</sup> Because IgE-mediated suppression works well in mice lacking these receptors,<sup>63</sup> it is unlikely that it is mediated via Fc receptors.

A unanimous finding is that high affinity IgG suppresses more efficiently than low-affinity IgG.<sup>36,60,67</sup> Primary and secondary IgG suppresses equally well in relation to their ability to bind to SRBC in an enzyme-linked immunosorbent assay (ELISA),<sup>68</sup> implying that the amount of IgG attached to the antigen, rather than the affinity per se, determines the suppressive capacity.

In summary, all murine subclasses are suppressive and high affinity IgG is more efficient than low-affinity IgG. The ability to suppress erythrocyte responses is not a unique feature of IgG antibodies although they are probably the most important ones owing to high affinity, high concentrations and long serum half life. The vast majority of our knowledge about antibody-mediated immune suppression stems from studies of IgG.

### 4 | CAN F(ab')<sub>2</sub> FRAGMENTS SUPPRESS?

Of crucial importance to understand the mechanism underlying IgG-mediated suppression is to determine whether it is dependent on the Fc region of IgG or not. Several laboratories have tested whether F(ab')<sub>2</sub> fragments can suppress, but results have been conflicting. Some investigators found that F(ab')<sub>2</sub> fragments do suppress<sup>26,38,47,69</sup> and others that they do not.<sup>36,42,56</sup> An inherent difficulty with these experiments is that F(ab')<sub>2</sub> fragments are eliminated faster than intact IgG owing to lack of binding to the neonatal FcR (FcRn) known to protect IgG from proteolysis.<sup>70</sup> However, the reason for the discrepancies has not yet been clarified.

### 5 | WHEN MUST IgG BE ADMINISTERED IN RELATION TO ANTIGEN IN ORDER TO SUPPRESS?

In most experimental systems in which immunosuppression is studied, IgG is administered one or a few hours before the antigen. However, there are many examples of

efficient suppression also when IgG is administered after the antigen. In Rhesus prophylaxis, anti-D administered to the mother 72 hours after delivery of the baby, works well.<sup>71</sup> In animal models, IgG can inhibit an ongoing antibody response as demonstrated by studying single cells producing IgM anti-SRBC.<sup>27,52,72,73</sup> For example, IgG administered 1-6 days after SRBC suppressed the direct plaque forming cell (PFC) response 11 days after immunization with SRBC, that is after having interacted with the antigen during 5-10 days.<sup>72</sup> In another experimental set-up, IgG administered 2-4 days after SRBC suppressed the PFC response 7 days after immunization with SRBC, after 3-5 days of interaction.<sup>52</sup> Also antibody responses to bacteriophages<sup>27</sup> and KLH<sup>26</sup> can be inhibited by IgG administered 1-3 days after immunization with antigen. These observations suggest that the antigen must continuously interact with the immune system in order for a sustained IgM response to take place. Possibly, passively administered IgG interferes with this interaction and terminates the antibody response.

## 6 | IS IgG-MEDIATED SUPPRESSION DEPENDENT ON FcγRs?

Because of the discrepant results regarding the ability of F(ab')<sub>2</sub> fragments to suppress, it was of interest to determine Fc dependence with alternative methods. Using various Fc receptor for IgG (FcγR)-deficient mice, several laboratories have reported that IgG suppresses efficiently in mice lacking the activating receptors FcγRI, FcγRIII, and FcγRIV (owing to lack of the common Fc receptor gamma chain, FcRγ)<sup>38,40,44,47,74</sup> or the inhibitory FcγRIIB.<sup>38,47,52,74</sup> Suppression was normal in double knockout mice lacking both FcRγ and FcγRIIB<sup>38</sup> as well as in mice lacking FcRn.<sup>38</sup> SRBC-specific IgG also suppresses antibody responses to SRBC efficiently in complement factor C3 (C3) × FcRγ double knockout mice.<sup>75</sup> In contrast, antibody responses to KEL-RBC were not suppressed by KEL-specific IgG in C3 × FcRγ double knockout mice.<sup>44</sup> With this exception,<sup>44</sup> IgG-mediated suppression works well in all complement (see below) and/or FcγR-deficient mice tested so far.

## 7 | IS IgG-MEDIATED SUPPRESSION DEPENDENT ON COMPLEMENT?

The Fc region of IgG is required for the ability to fix complement factor C1q (C1q) and activate the classical complement pathway. It is feasible that lysis of erythrocytes may render them less immunogenic than intact cells or that increased

phagocytosis of IgG/complement-opsonized erythrocytes would make the antigen unavailable to B cells. Complement dependence of suppression has been addressed in a few studies. Monoclonal IgG1 antibodies, which could not activate complement, were efficient suppressors<sup>60,76</sup> and IgG suppressed antibody responses to SRBC in C1q, C3 and CR1/CR2 knockout mice.<sup>74</sup> In analogy, KEL-specific IgG suppressed IgG responses in C3 knockout mice immunized with KEL-RBC,<sup>44</sup> although, as mentioned above, IgG could not suppress in mice lacking both C3 and activating FcγRs.<sup>44</sup> In summary, the ability of IgG to activate complement cannot be the exclusive explanation for its ability to suppress antibody responses.

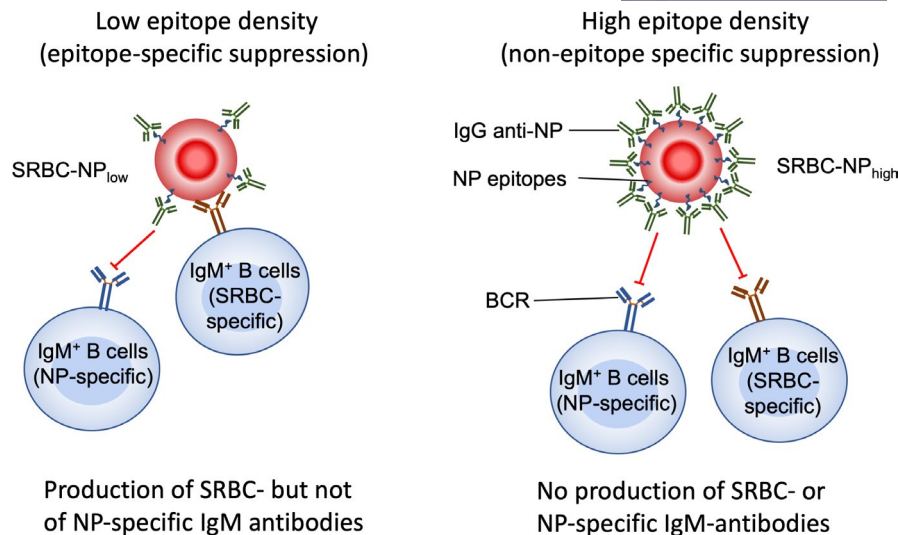
## 8 | IS IgG-MEDIATED SUPPRESSION EPITOPE SPECIFIC?

Suppression by IgG is antigen-specific in the sense that only responses to the antigen particle to which IgG binds are suppressed. This has been determined after administration to mice of IgG anti-SRBC + SRBC + horse red blood cells (HRBC), an antigen which does not crossreact with SRBC. Such studies show that the response to HRBC is unperturbed while the response to SRBC is suppressed.<sup>37,38</sup>

Whether IgG suppresses responses only to the epitope it binds to, or also to other epitopes on the same antigen, is an important question. Epitope specificity would indicate that, for example, epitope masking explains suppression while non-epitope specificity would indicate an Fc-dependent mechanism such as lysis, clearance or FcγRIIB-mediated inhibition of B-cell activation. However, both epitope-specific<sup>39-41,50,53,77</sup> and non-epitope-specific suppression<sup>36-39,41,42,46,48,76</sup> have been demonstrated and it has therefore been difficult to draw mechanistic conclusions from this type of experiments.

When we found that IgG suppressed efficiently in mice lacking all known FcγRs,<sup>38</sup> suggesting Fc-independence, it became important to understand how non-epitope-specific suppression, suggesting Fc dependence, could take place. We hypothesized that the discrepant results regarding epitope specificity may be explained by taking epitope density into account<sup>38</sup> (Figure 2). When IgG binds to an epitope present at low density, it would only suppress the antibody response to that epitope by masking it for B cells. The result would be epitope-specific suppression. When IgG binds to an epitope present at high density, it would suppress the antibody response both to that epitope and to neighbouring epitopes by sterically hindering B cells from accessing both types of epitopes. The result would be non-epitope-specific suppression. Experimental support for this hypothesis is that hapten-specific IgG, passively administered together with high- or low hapten density SRBC, suppressed the response





**FIGURE 2** The importance of epitope density for specificity of suppression. When IgG binds to an epitope present at low density, only the epitopes to which IgG binds will be hidden from B cells while other epitopes will be available for B-cell recognition. This results in epitope-specific suppression (left). When IgG binds to an epitope present at high density, both the epitopes to which IgG binds and neighbouring epitopes will be hidden from B cells. This results in non-epitope-specific suppression (right). Modified from Xu, H. et al, Scientific Reports 2018, 8:15292

against the hapten regardless of the epitope density<sup>39,41</sup> but suppressed the response against SRBC determinants only when high hapten density SRBC was used as antigen.<sup>39,41,60</sup> In line with this, suppression was epitope specific when murine erythrocytes, expressing both HOD and KEL, were used and it could be directly shown in superresolution microscopy that anti-KEL did not sterically hinder binding of anti-hen egg lysozyme (HEL) and vice versa.<sup>50</sup>

The requirement for high epitope density in order for IgG to induce non-epitope-specific suppression is compatible with earlier experiments in which suppression of the entire antibody response to SRBC was studied. The ability of a panel of monoclonal SRBC-specific IgG antibodies to suppress SRBC responses correlated with their binding plateau to SRBC, and therefore with epitope density,<sup>37</sup> and the ability of primary and secondary IgG to suppress correlated with their level of binding to SRBC.<sup>68</sup> Moreover, a mixture of monoclonal IgG antibodies recognizing different epitopes on SRBC<sup>37,78</sup> or on KEL-RBC<sup>79</sup> was more suppressive than each monoclonal by itself, pointing to an additive effect.

With one exception,<sup>50</sup> the observations discussed above concern suppression of IgM responses which has been studied in much greater detail than suppression of IgG responses. For unknown reasons, suppression of IgG responses appears to be epitope specific both when high and low density of NP-SRBC are used.<sup>40,41</sup>

In summary, IgG-mediated suppression is antigen-specific, that is, only responses to the antigen to which IgG binds are suppressed. When IgG binds to an epitope present at low density, suppression is epitope specific. However, several lines of evidence suggest that when IgG binds to an epitope present at high density, it will suppress the response also to

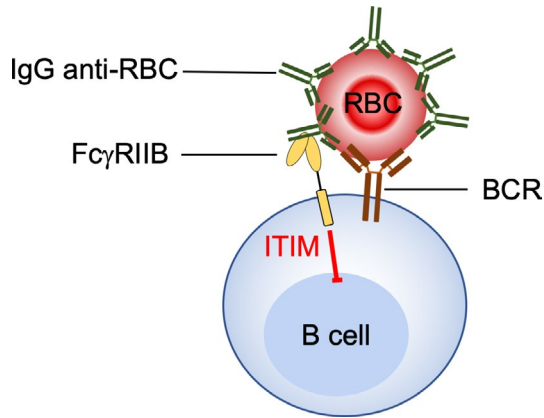
neighbouring epitopes. Therefore, the existence of non-epitope-specific suppression cannot be taken as evidence for Fc-dependent suppression.

## 9 | HYPOTHESES TO EXPLAIN IgG-MEDIATED SUPPRESSION

The major hypotheses that have been proposed to explain the suppressive effect of IgG are (a) central inhibition of B-cell activation through co-crosslinking of BCR with the negatively regulating FcγRIIB by IgG-antigen complexes, (b) complement-mediated lysis of red blood cells, (c) increased clearance of IgG-antigen complexes, (d) masking of epitopes, preventing B cells from binding, and (e) trogocytosis, causing loss of IgG-bound epitopes, preventing B cells from binding. It cannot be excluded that several mechanisms contribute to suppression, but the discussion below will be based on the assumption that one single mechanism plays a dominant role.

### 9.1 | Can FcγRIIB-mediated central inhibition of B cells explain IgG-mediated suppression?

One hypothesis for how IgG suppresses antibody responses has been that the IgG-antigen immune complexes co-crosslink the B-cell receptor and the negatively regulating FcγRIIB. This receptor contains immunoreceptor tyrosine-based inhibition motifs (ITIM) in its intracytoplasmic domain and can inhibit activation of receptors containing immunoreceptor tyrosine-based activation motifs (ITAM), such as the Igα/Igβ



**FIGURE 3** Central inhibition of B-cell activation. IgG-antigen complexes may co-crosslink BCR with the negatively regulating FcγRIIB. This would lead to inhibition of the specific B cells and suppressed antibody responses

of the BCR. This would lead to central inhibition of antigen-specific B cells because only cells with receptors for the antigen in question would be able to bind to the IgG-antigen complex and thus be downregulated (Figure 3). There is ample evidence that FcγRIIB indeed exerts a negative impact on many types of immune responses mediated via immunoreceptors which signal via ITAM (reviewed in Ref80). One example is the 'enhanced enhancement' of antibody responses to IgG-complexed proteins observed in FcγRIIB knockout mice.<sup>30,34</sup> As mentioned in the introduction, IgG-mediated feedback enhancement operates via activating FcγRs, which signal via ITAM. In the absence of FcγRIIB, the enhancement is unregulated and becomes even more pronounced than in wildtype mice, thus demonstrating an *in vivo* effect of FcγRIIB.<sup>30,34</sup>

Nevertheless, negative regulation by FcγRIIB cannot explain feedback suppression by passively administered IgG. This is clearly shown by the unperturbed suppression of responses to SRBC<sup>38,52,74</sup> or HOD-RBC<sup>47</sup> in mice lacking FcγRIIB. These observations may seem paradoxical, given the well-documented negative regulatory effect of FcγRIIB, but become logical assuming that the role of this receptor is to downmodulate rather than to completely prevent a B-cell response. The initial studies of suppression in mice lacking FcγRIIB, activating FcγRs, or both, showed that IgG, administered in close temporal relation to SRBC or SRBC-TNP, suppressed the IgM anti-SRBC response equally well as in wildtype mice.<sup>38</sup> This finding argued against the current paradigm stating that suppression was Fc-dependent, and it was suggested that the outcome would have been different under other experimental conditions. In a follow-up study, it was shown that IgG efficiently suppressed early IgG responses and secondary antibody responses in FcγRIIB knockout mice, and that IgG administered up to 4 days after SRBC suppressed equally well in FcγRIIB knockout and wildtype mice.<sup>52</sup> Later, long-term

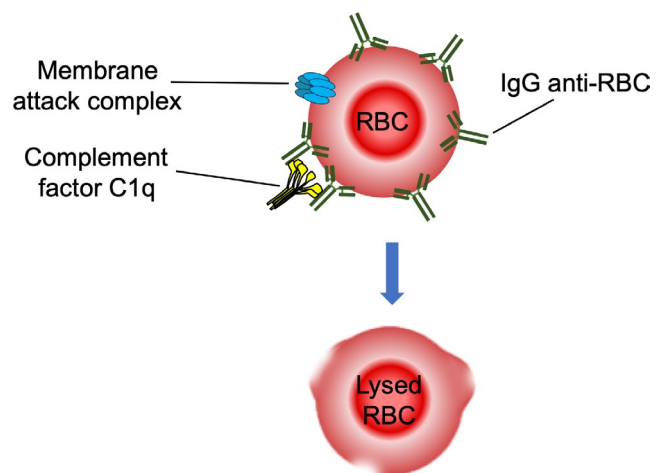
IgG responses were also shown to be suppressed by IgG in FcγRIIB knockout mice.<sup>74</sup>

Monoclonal IgG3,<sup>36,37</sup> IgE<sup>38,52</sup> and IgM<sup>36</sup> as well as polyclonal IgM<sup>7,62</sup> can suppress antibody responses to SRBC. Neither IgG3<sup>61</sup> nor IgM binds to FcγRIIB. IgE has been reported to do so<sup>64</sup> but suppresses well also in FcγRIIB knockout mice.<sup>63</sup> Unless a different mechanism for suppression by IgG3, IgE and IgM than for suppression by IgG1, IgG2a and IgG2b is postulated, these observations argue against involvement of FcγRIIB in suppression.

In summary, there is no evidence supporting a role for FcγRIIB in IgG-mediated immune suppression of antibody responses to erythrocytes *in vivo* although the receptor negatively regulates other types of immune responses.

## 9.2 | Can complement-mediated lysis of erythrocytes explain IgG-mediated suppression?

Most IgG subclasses can activate complement and activation of complement by IgG binding to erythrocyte surfaces may lead to haemolysis and thereby loss of immunogenicity (Figure 4). However, the ability of non-complement activating IgG to suppress<sup>60,76</sup> and the efficient suppression in mice lacking either C1q<sup>74</sup> or C3<sup>44,74</sup> suggest that suppression does not operate via lysis. Moreover, epitope specificity of suppression<sup>39-41,50,53,77</sup> is hard to reconcile with lysis which would destroy the entire cell. The fact that IgG can suppress antibody response to viruses<sup>27,28</sup> and

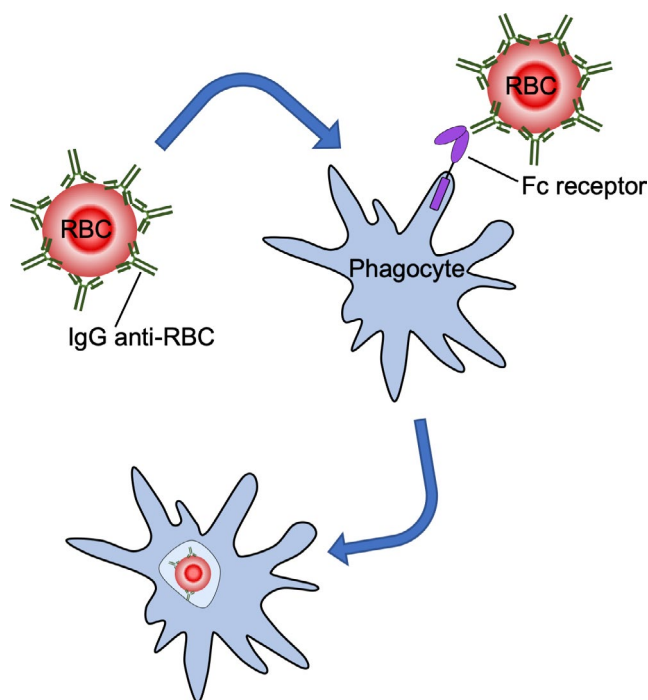


**FIGURE 4** Complement-mediated lysis. IgG binding to an erythrocyte may fix C1q and initiate the classical complement pathway resulting in formation of membrane attack complexes on the erythrocyte surface. This would lead to haemolysis of the erythrocyte through osmotic shock, possibly followed by reduced immunogenicity and suppressed antibody responses

to proteins in adjuvants,<sup>25,26</sup> which are not susceptible to haemolysis, also argues against a role of complement. In favour of a partial contribution of complement is the observation that suppression does not work in C3  $\times$  FcR $\gamma$  double knockout mice immunized with KEL-RBC.<sup>44</sup> Notably, suppression works well in single C3 knockout mice<sup>44</sup> and IgG suppresses efficiently in C3  $\times$  FcR $\gamma$  double knockout mice immunized with SRBC.<sup>75</sup> In summary, a dominant role for complement-mediated lysis or complement-dependent clearance in IgG-mediated immune suppression seems unlikely.

### 9.3 | Can clearance/phagocytosis/relocalization of antigen explain IgG-mediated suppression?

Increased clearance of IgG-erythrocyte complexes has frequently been discussed as an explanation for IgG-mediated immune suppression. Elimination of erythrocytes from the circulation, and as a consequence from secondary lymphoid organs, would prevent recognition by specific B cells and result in a lack of antibody responses (Figure 5).



**FIGURE 5** Clearance of IgG-antigen complexes. Antigen may be opsonized by specific IgG (and complement components when IgG is able to activate complement). The IgG-antigen complexes would then be efficiently eliminated by phagocytosis, leading to rapid clearance of the antigen from the circulation. This would prevent antigen from reaching secondary lymphoid organs where the immune responses take place

#### 9.3.1 | Xenogeneic erythrocytes and clearance

Xenogeneic erythrocytes, for example SRBC, administered intravenously are completely eliminated from the blood within ten minutes whether IgG is co-administered or not. Already after one minute, the levels of SRBC in the blood are similar in groups with and without passively administered IgG<sup>39</sup> making it unlikely that IgG-mediated clearance is responsible for suppression in these experimental systems. The conclusion is strengthened by the observations that antibody responses can be suppressed when IgG is administered several days after SRBC,<sup>8,52,53,72,73</sup> that is at a time point when no antigen remains in the circulation. Moreover, macrophages fed with IgG-SRBC and injected iv to mice induced a higher, rather than a lower, response than macrophages fed with SRBC alone.<sup>54</sup>

Most likely, clearance induced by IgG would be dependent on Fc $\gamma$ Rs and/or complement. Therefore, the findings that IgG suppresses SRBC responses in mice lacking activating Fc $\gamma$ Rs,<sup>38,40,47,74</sup> C1q or C3,<sup>74</sup> or both C3 and activating Fc $\gamma$ Rs,<sup>75</sup> argue against clearance as an important mechanism in this experimental system. The antibody response against intravenously administered SRBC is primarily initiated in the spleen and passively administered IgG has been shown to decrease the amount of SRBC in the marginal zone of wild-type mice.<sup>39,40</sup> In FcR $\gamma$  knockout mice, IgG did not decrease the amount of SRBC in the marginal zone, but nevertheless suppressed the antibody response to the same degree as in wildtype mice.<sup>40</sup> This implies a lack of correlation between suppression and the ability of IgG to decrease clearance as detected by antigen localization to the spleen.

Epitope specificity of suppression<sup>39-41,53,77</sup> is difficult to reconcile with clearance because the entire erythrocyte should be cleared and suppression/lack of response should affect all epitopes. Compatible with clearance as an explanation for IgG-mediated suppression are observations pointing to Fc dependence, such as lack of suppression by F(ab')<sub>2</sub> fragments<sup>36,42,56</sup> and non-epitope specificity of suppression.<sup>36-39,41,42,46,48,76</sup> However, as discussed elsewhere in this review, there may be alternative explanations for these findings.

#### 9.3.2 | Allogeneic erythrocytes and clearance

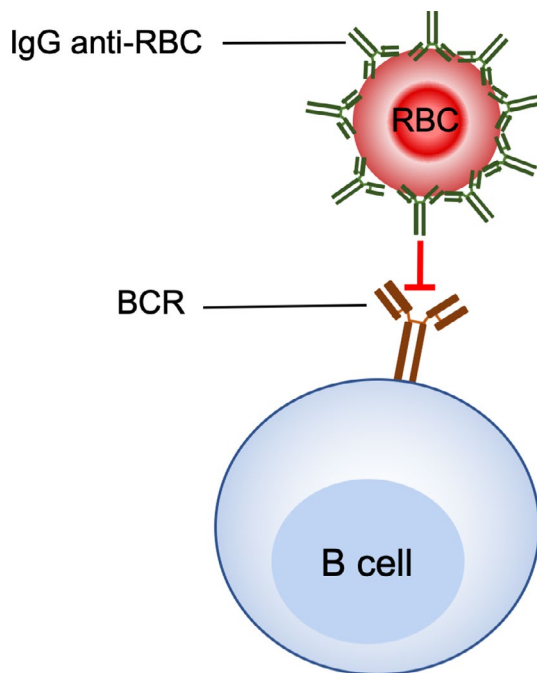
Because allogeneic erythrocytes persist much longer in the circulation than xenogeneic erythrocytes, the effects on clearance by IgG can be directly studied in these experimental systems. Also here, a number of observations argue against a major role for clearance. One example is experiments showing that three monoclonal IgG antibodies specific for HOD-RBC all suppressed the HEL-specific antibody response although none of them increased clearance.<sup>46</sup> Efficient suppression in spite of poor clearance has also been reported

with polyclonal IgG in allogeneic murine systems<sup>43,44,48,50</sup> as well as with anti-RhD in humans.<sup>81-83</sup>

In summary, the arguments against an exclusive role for clearance in IgG-mediated immune suppression are strong. There are many examples of efficient suppression in the total or partial absence of clearance, both in murine and human allogeneic systems. In xenogeneic systems, the strongest argument against clearance is that suppression works when IgG is administered several days after the antigen although erythrocytes are cleared from the circulation within minutes even without passive transfer of IgG. This does not exclude that clearance may have an additive effect to other mechanisms, at least when allogeneic erythrocytes are used.

#### 9.4 | Can epitope masking explain IgG-mediated suppression?

Another hypothesis to explain IgG-mediated suppression is epitope masking. In this model, passively injected IgG would prevent antibody responses to antigen simply by hiding the epitopes from recognition by cognate B cells (Figure 6). This model relies only on the binding between antibody and antigen and would be independent of the Fc region of IgG. It is difficult to directly prove epitope masking and it remains primarily an 'exclusion diagnosis' relying on whether data are compatible with the hypothesis or not.



**FIGURE 6** Masking of epitopes. Passively transferred IgG binds to its specific epitopes and masks them. This prevents specific B cells from recognizing and binding to the epitope (and also to neighbouring epitopes during high density conditions) and results in a suppressed antibody response

The fact that  $F(ab')_2$  fragments,<sup>26,38,47,69</sup> IgM<sup>7,36,62</sup> and IgE<sup>38,63</sup> can suppress is to be expected should masking of epitopes explain the phenomenon. Compatible with epitope masking is also the efficient suppression seen during conditions when IgG cannot activate complement<sup>60,76</sup> or in the absence of C1q or C3<sup>44,74</sup> or FcγRs.<sup>38,40,44,47,52,74</sup> A strong argument in favour of epitope masking is the ability of IgG to suppress antibody responses to SRBC in double knockout mice lacking both C3 and activating FcγRs.<sup>75</sup>

As would be predicted from the epitope masking model, specific T helper cell responses are normal in mice in which the antibody responses are efficiently suppressed by IgG.<sup>28,38,39,54</sup> IgG, masking B-cell epitopes on the antigen surface, would not prevent the IgG-erythrocyte or IgG-virus complexes from being taken up and presented to T helper cells by antigen-presenting cells.

Two independent studies have shown that mice immunized with IgG anti-SRBC and SRBC produced antibodies to the Fc region of IgG but, as expected, not to SRBC.<sup>84,85</sup> This is what would be anticipated should the passively administered IgG, bound to the SRBC surface, hide SRBC epitopes but present their Fc regions to B cells. Moreover, the additive suppressive effect of a blend of monoclonal antibodies specific for different epitopes on the erythrocyte fits with epitope masking<sup>37,78,79</sup> as does the finding that suppression correlates with antibody affinity.<sup>36,60,67</sup>

Epitope specificity of suppression<sup>39-41,53,77</sup> is of course to be expected during epitope masking. As discussed above, also non-epitope-specific suppression may fit into the model provided that epitope density is considered.

While the above findings are all compatible with the epitope masking hypothesis, other findings are not. In studies using murine allogeneic erythrocytes as antigen, suppression was shown to occur in the apparent absence of epitope masking.<sup>46,48</sup> Moreover, the inability of  $F(ab')_2$  fragments to suppress<sup>36,42,56</sup> and the inability of immune serum to suppress responses to allogeneic erythrocytes in mice lacking both C3 and activating FcγRs<sup>44</sup> are incompatible with epitope masking. However, for the latter observations discrepant findings have been reported:  $F(ab')_2$  sometimes suppresses<sup>26,38,47,69</sup> and IgG can suppress responses to SRBC in C3 × FcγR double knockout mice.<sup>75</sup>

In summary, many observations are compatible with the epitope masking hypothesis although conclusive experiments are lacking. Some observations are incompatible with epitope masking, but for several of them, either alternative explanations or conflicting results have been reported.

#### 9.5 | Can trogocytosis (antigen modulation or antigen loss) explain IgG-mediated suppression?

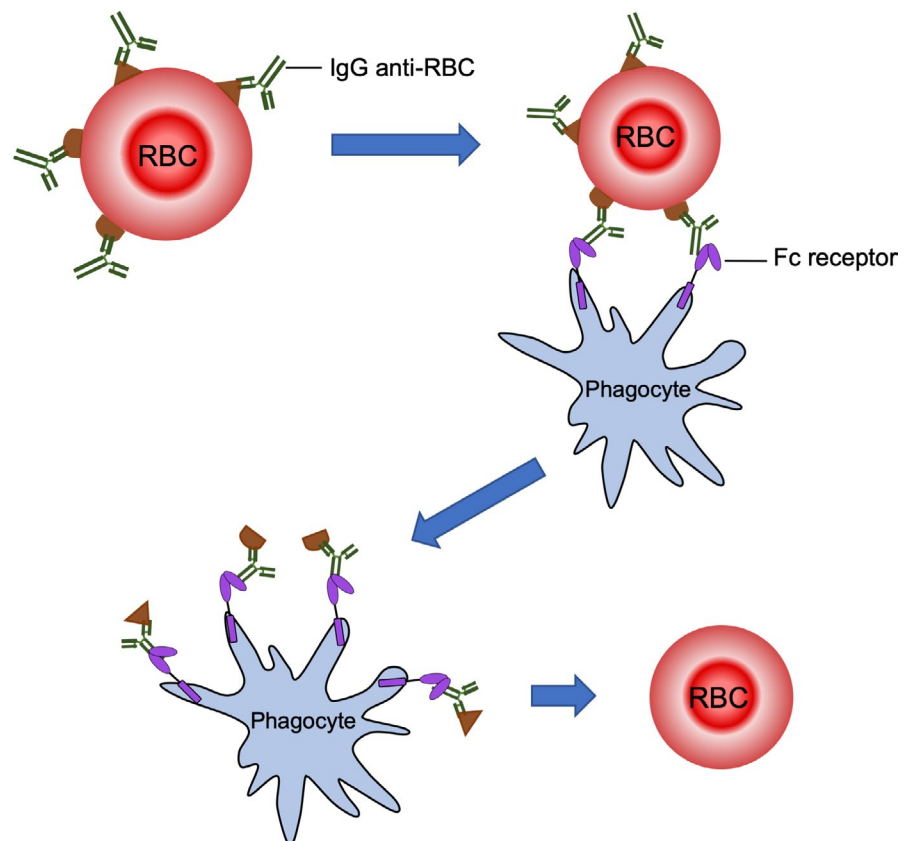
Trogocytosis is a process through which cell membrane fragments are transferred from one cell to another after the two



cells have formed an immunologic synapse.<sup>86,87</sup> Recently, this mechanism, also referred to as antigen modulation or antigen loss, has been suggested to explain IgG-mediated immune suppression in experimental systems using mouse allogeneic erythrocytes, expressing transgenic epitopes, as antigen.<sup>43,44,48–50</sup> The idea is that IgG, binding to a certain epitope on an erythrocyte, induces removal of that epitope from the cell surface resulting in lack of an immune response (Figure 7). It has been shown by flow cytometry, and sometimes also by Western blot analysis, that the epitope to which the suppressing antibody bound was lost from the erythrocyte membrane.<sup>44,50</sup> Mice do not have blood groups and do not produce antibodies against the allogeneic erythrocytes per se. Therefore, only responses against the transgenic epitopes, HOD or KEL, can be measured in these models. The mechanism behind trogocytosis is incompletely understood. In studies of IgG-induced antigen modulation, in which suppression of antibody responses was not analysed in parallel, modulation was either independent<sup>88,89</sup> or dependent<sup>45,90</sup> on FcγR receptors. Other studies showed dependence on C3<sup>89</sup> or suggested antigen-antibody crosslinking on the erythrocyte surface as the mechanism.<sup>88</sup> When modulation and suppression were studied in parallel, both worked in the absence of either FcγRs or C3<sup>44,49</sup> but not in the simultaneous absence of both.<sup>44</sup> The discrepant results concerning the mechanism behind trogocytosis make it difficult to relate to other observations of Fc dependence or independence of IgG-mediated immune suppression.

Epitope specificity has been studied also in the allogeneic models. Using mouse erythrocytes expressing both KEL and HOD, it was found that anti-KEL suppressed KEL- but not HEL-specific responses and anti-HEL suppressed HEL- but not KEL-specific responses.<sup>50</sup> This suggested that suppression was epitope specific and it was shown by super resolution microscopy that anti-KEL antibodies did not sterically hinder the binding of anti-HEL to the HOD determinant and vice versa.<sup>50</sup> Non-epitope-specific suppression has been described in experiments where anti-OVA suppressed the anti-HEL response to HOD-RBC.<sup>48</sup> Possibly, these results may be explained by the close proximity of OVA, HEL and Duffy determinants on the transgenic epitope causing anti-OVA to modulate/remove all epitopes.

The observations described above are compatible with trogocytosis as the mechanism behind antibody-mediated suppression. Other findings are hard to fit into this model, such as suppression of antibody responses against viruses<sup>27,28</sup> and proteins<sup>25,26</sup> which do not have a cell membrane. The complete suppression of the antibody response against all epitopes on an erythrocyte is also difficult to understand in these terms, since it would require that the entire cell membrane is removed by trogocytosis. In some situations, both when xenogeneic<sup>41,53</sup> and allogeneic<sup>50</sup> erythrocytes were used, IgG suppressed the response to the specific epitope but enhanced the response to other epitopes on the same antigen. It is not clear how trogocytosis would accomplish this. These



**FIGURE 7** Trogocytosis/antigen modulation. Passively transferred IgG binds to its specific epitopes. This may lead to loss of these epitopes, possibly when phagocytes expressing FcγR recognize the IgG-erythrocyte complex and removes the IgG-bound epitopes

observations may be better explained with epitope masking, where unmasked epitopes could be efficiently presented to T helper cells and induce an enhanced antibody response. As mentioned in the Introduction, IgG in complex with its antigen can feedback enhance T helper cell and antibody responses by increasing uptake and presentation of the antigen by antigen-presenting cells via their FcγRs. This has primarily been described for proteins, but may also affect erythrocyte epitopes which are not masked by IgG.

In summary, experiments using transgenic allogeneic erythrocytes as antigen have demonstrated that suppression of the antibody response to the transgenic epitope coincides with its disappearance from the erythrocyte surface. These observations suggest that trogocytosis is involved in IgG-mediated suppression. The mechanism

behind trogocytosis is poorly understood and many basic questions are left to be solved such as the dependence on complement and FcγRs.

## 10 | CONCLUDING REMARKS

It has proven difficult to conclusively determine the mechanism(s) through which passively administered IgG suppresses the specific antibody response. In Table 1, a number of the experimental observations discussed above are listed. An attempt has been made to determine whether each of these can be fitted into the various hypotheses (columns 1-5) as well as whether they are compatible with dependence on the Fc region of IgG (column 6). Notably, the 'yes' and

**TABLE 1** Compatibility of experimental observations with various hypotheses as the single/major explanation for IgG-mediated suppression

Observations	Inhibition by FcγRIIB	CDC <sup>a</sup>	Clearance/ Phagocytosis	Epitope masking	Trogocytosis <sup>b</sup>	IgG(Fc) is required
Epitope specificity <sup>39-41,50,53,77</sup>	NO	NO	NO	Yes	Yes	NO
Non-epitope specificity <sup>36-39,41,42,46,48,76</sup>	Yes	Yes	Yes	Yes <sup>c</sup>	Yes <sup>c</sup>	NO <sup>c</sup>
F(ab') <sub>2</sub> can suppress <sup>26,38,47,69</sup>	NO	NO	NO <sup>d</sup>	Yes	NO	NO
F(ab') <sub>2</sub> cannot suppress <sup>36,42,56</sup>	Yes	Yes	Yes	NO	Yes	Yes
IgM can suppress <sup>7,36,62</sup>	NO	Yes	Yes	Yes	NO	NO
IgE can suppress <sup>38,63</sup>	NO	NO	? <sup>e</sup>	Yes	Yes	NO
Mouse IgG3 can suppress <sup>36,37</sup>	NO	Yes	Yes	Yes	Yes	Yes
High affinity IgG suppresses better than low <sup>36,60,67</sup>	Yes	Yes	Yes	Yes	Yes	Yes
Additive effect of mAb blends <sup>37,78,79</sup>	Yes	Yes	? <sup>e</sup>	Yes	Yes	Yes
Correlation between epitope density and suppression <sup>37,39,41,60,68</sup>	Yes	Yes	? <sup>e</sup>	Yes	Yes	Yes
Works in FcγRIIB KO <sup>38,47,52,74</sup>	NO	Yes	Yes	Yes	Yes	NO
Works in FcγR KO <sup>38,40,44,47,74</sup>	Yes	Yes	Yes	Yes	NO	NO
Works in FcγRIIBxFcγR dKO <sup>38</sup>	NO	Yes	Yes	Yes	NO	NO
Works in FcRn KO <sup>38</sup>	Yes	Yes	Yes	Yes	Yes	NO
Works in C3 KO <sup>44,74</sup>	Yes	NO	Yes	Yes	Yes	NO
Works in C1q KO <sup>74</sup>	Yes	NO	Yes	Yes	Yes	NO
Non-C-activating IgG can suppress <sup>60,76</sup>	Yes	NO	Yes	Yes	Yes	NO
Works in (C3xFcγR) double KO <sup>75</sup>	Yes	NO	NO <sup>d</sup>	Yes	NO	NO
Does not work in (C3xFcγR) double KO <sup>44</sup>	Yes	Yes	Yes	NO	Yes	Yes
No correlation with clearance <sup>43,44,48,50,81-83</sup>	Yes	Yes	NO	Yes	Yes	Yes
IgG administered after Ag can suppress <sup>8,27,52,53,71-73</sup>	Yes	NO	NO/yes <sup>f</sup>	Yes	? <sup>e</sup>	NO/yes <sup>f</sup>
Poor suppression of CD4+ T cells <sup>28,38,39,54,58</sup>	Yes	Yes	Yes	Yes	Yes	Yes

<sup>a</sup>Complement-dependent cytotoxicity.

<sup>b</sup>Yes or NO is based on the assumption that trogocytosis depends on activating FcγRs, although this is not definitely proven.

<sup>c</sup>During high epitope density.

<sup>d</sup>Assuming that clearance is dependent on the IgG(Fc) region.

<sup>e</sup>Not sufficiently studied in this context for a yes or no.

<sup>f</sup>No: when Ag is rapidly eliminated (eg xenogeneic erythrocytes); Yes: when antigen remains for longer times in the circulation (eg allogeneic erythrocytes).

'no' notations are based on the idea that the model in question is the single/major explanation for suppression. As can be seen from Table 1, many observations are compatible with several hypotheses, and therefore, the number of 'yes' is not very helpful in differentiating between them. More informative may be to, à la Karl Popper, try to falsify the hypotheses and study the 'no's'. Suppression works well in mice lacking FcγRIIB or complement factors, and these findings strongly argue against a role of negative B-cell regulation via FcγRIIB or complement-mediated lysis in IgG-mediated suppression. Suppression has also been shown to work in the absence of demonstrable clearance, and therefore, a major role for this mechanism is unlikely. An overwhelming number of observations are incompatible with requirement for the Fc region of IgG for induction of suppression (Table 1, right column).

The remaining hypotheses are epitope masking and trogocytosis. In other words, does IgG suppress by hiding epitopes from B cells or by snatching epitopes from the erythrocytes thereby preventing B-cell recognition? There are few 'no's' for either of these two hypotheses. Although the mechanism behind trogocytosis is not fully understood, we have assumed in the table that it is Fc-dependent. In the SRBC model, the experimental support for a major role of epitope masking is strong. Suppression works in the absence of (a) the Fc region of IgG (F(ab')<sub>2</sub>, IgE, IgM are suppressive), (b) FcRn, the inhibitory FcγRIIB, activating FcγRI, FcγRIII and FcγRIV, (c) both inhibitory and activating FcγRs, (d) C3, C1q, CR1/CR2, (e) both C3 and the activating FcγRI, FcγRIII and FcγRIV. Moreover, suppression is epitope specific, except when IgG binds to the SRBC surface at a high density when suppression of responses also to neighbouring epitopes can be seen. T helper cell induction is normal although the antibody responses are suppressed. There is a correlation between suppression and the amount of IgG bound to the SRBC surface. IgG administered several days after SRBC, when all antigen is cleared from the circulation, can suppress. An argument against epitope masking is the inability of F(ab')<sub>2</sub> fragments to suppress, but the ability of IgG to suppress anti-SRBC responses in C3 × FcRγ double knockout mice strongly supports that suppression is independent of the Fc region. To our knowledge, trogocytosis has not been investigated in the SRBC system, and it seems unlikely that the observed complete suppression of responses to all epitopes on the SRBC would be caused by this mechanism. In models using transgenic mouse erythrocytes, trogocytosis was proposed to explain IgG-mediated immune suppression from the observation that suppression coincided with loss of epitopes bound by the suppressive IgG. This hypothesis is quite recent and the mechanism behind IgG-induced trogocytosis is not yet well understood. Clearly, there are differences between how xenogeneic and

allogeneic erythrocytes are handled by the immune system. The most important difference is probably the slower clearance of allogeneic erythrocytes than of xenogeneic erythrocytes. Another difference seems to be whether suppression operates in the simultaneous absence of both FcRγ and C3. There are also similarities, such as the independence of activating and inhibitory FcγRs or complement for suppression, found in all xenogeneic and some allogeneic studies. Also, more efficient suppression by a mixture of monoclonal antibodies than by each individual monoclonal is reported in both systems. Future studies will hopefully elucidate whether suppression of antibody responses to SRBC and to allogeneic erythrocytes is caused by different mechanisms or whether current differences can be explained by different experimental set-ups.

Determining how small amounts of IgG can completely suppress, or perhaps more correctly put, prevent, an antibody response is of great theoretical interest. It is also of practical importance. Rhesus prophylaxis currently relies on IgG antibodies obtained from polyclonal human sera. Understanding which qualities in an IgG antibody that are important for suppression would facilitate the search for clinically useful monoclonal IgG anti-RhD antibodies.

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## CONFLICT OF INTEREST

Authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

HX and BH wrote the manuscript and prepared the figures.

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