Feedback Enhancement of Antibody Responses via Complement and Fc Receptors

BY

JÖRGEN DAHLSTRÖM
Abstract


IgG, IgM and IgE in complex with antigen have the capacity to regulate specific immune responses. In this investigation, the role of Fc receptors for IgG (FcγRI, FcγRII and FcγRIII) and complement receptors 1 and 2 (CR1/2) for antibody-mediated enhancement of antibody responses are investigated.

IgM is known to efficiently activate complement and thereby enhance specific antibody responses but it is not known if this involves binding to CR1/2. Using CR1/2 deficient mice, immunized with sheep erythrocytes alone or together with specific IgM, we present evidence that IgM-mediated enhancement is completely dependent on CR1/2 expression, whereas IgG or IgE in complex with bovine serum albumin (BSA) induce strong antibody responses in CR1/2-deficient mice. Enhancement by IgE is mediated via the low affinity receptor for IgE (FcεRII, CD23). However, the receptors which are involved in IgG-mediated enhancement are not known. We find that γ-chain-deficient mice (lacking FcγRI and FcγRIII) have impaired antibody responses to IgG/BSA complexes. In contrast, FcγRIII-deficient mice have normal responses, suggesting that FcγRI mediates the effect. Furthermore, IgG/BSA complexes induce up to 189-fold stronger antibody responses in FcγRIIB-deficient mice than in wild-type mice. The threshold dose of IgG/BSA required was lower, the response was sustained for longer and initiated earlier in FcγRIIB-deficient than in wild-type animals. The findings suggest that FcγRIIB acts as a "safety-valve" preventing excessive antibody production during an immune response. We show for the first time that IgG3/BSA complexes can mediate enhancement of specific antibody responses. Their effect does not involve known Fcγ receptors.

Key Words: Mouse, transgenic/knockout, immune regulation, B lymphocytes, Fc receptors, complement, IgG, IgM, IgE, FcγRI, FcγRII, FcγRIII, CR1, CR2, CD21, CD35.

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ISSN 0282-7476
ISBN 91-554-4943-3

Printed in Sweden by Lindbergs Grafiska HB, Uppsala 2001
To my wife Sara
and my daughter Johanna
This thesis is based on the following articles, which are referred to in the text by their roman numerals:

I Applequist, S.E., Dahlström, J., Jiang, N., Molina, H. and Heyman, B. 2000. Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgG/Ag and IgE/Ag, but not IgM/Ag complexes. *J. Immunol.* 165:2398-2403.


III Dahlström, J., Wernersson, S. and Heyman, B. FcγRIIB negatively regulates Ab responses to IgG/Ag, but not IgE/Ag or IgM/Ag complexes. *Submitted.*


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Abbreviations

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<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
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<td>APC</td>
<td>antigen presenting cell</td>
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<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CFA</td>
<td>complete Freund's adjuvant</td>
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<tr>
<td>CR</td>
<td>complement receptor</td>
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<td>CVF</td>
<td>cobra venom factor</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>enzyme-linked immunospot assay</td>
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<tr>
<td>FcR</td>
<td>receptor for the Fc portion of an antibody</td>
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<tr>
<td>FcRγ</td>
<td>common γ-chain associated with FceRI, FcγRI and FcγRIII</td>
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<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>GC</td>
<td>germinal center</td>
</tr>
<tr>
<td>HRBC</td>
<td>horse red blood cells</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>IC</td>
<td>immune complex</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>KLC</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MBL</td>
<td>mannan-binding lectin</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PFC</td>
<td>plaque forming cell</td>
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<tr>
<td>Rh</td>
<td>rhesus</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>SRBC</td>
<td>sheep red blood cells</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNP</td>
<td>2, 4, 6-trinitrophenyl</td>
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Introduction

The ability of foreign agents (referred to as antigens (Ags)), such as bacteria, parasites and viruses, to invade our bodies and cause great damage has led to the evolution of a sophisticated defense system, the immune system. This system is a network composed of several soluble and cellular components that have as their sole objective to protect the body from infectious diseases. The immune system has the ability to adapt and specifically "remember" the Ags to which it has been exposed. A central role of the specific immune response is production of specific antibodies (Abs). The Abs have the capacity to bind foreign substances and promote processes subsequently leading to elimination of the Ags. These reactions probably occur continuously, with no ill effect on the host. However, the adaptability and specificity of sufficient amounts of Abs is a slow process. Therefore we also have a more rapid system, the innate immune system. The innate immune system involves engulfing of Ags by phagocytic cells or elimination by protein components (complement molecules) which have evolved to recognize certain common patterns found among pathogens. The combination of innate and adaptive immunity completes the host defense without which we would be unable to survive.

Specific immunity is generated by two cell types, B and T cells, that can recognize foreign Ags. Whereas the B cell response is characterized by production of specific Abs, T cells have several functions including activation of B cells, production of soluble mediators (cytokines) and killing of infected cells by lysis. The native Ag is recognized by mature B cells via surface-bound immunoglobulin (sIg), of the IgD or IgM type, associated with an Igα/Igβ complex. sIg and Igα/Igβ is also called the B cell receptor (BCR). Activation of B cells is classically divided into two steps: "signal one" is provided when the BCR binds the Ag and "signal two" is provided by the interaction with specific T cells and their cytokines. However, bacterial Ags, such as lipopolysaccharide, can stimulate B cells to produce Abs in the absence of T cell "help" and are therefore called T cell-independent Ags. Ags that are unable to stimulate B cells without T cell "help" are called T cell-dependent Ags.

Whereas B cells recognize Ag in its native form, T cells are unable to do this. Instead, T cells, via their T cell receptor (TCR), bind Ag that has been processed and presented in the form of peptides bound to major histocompatibility complex molecule (MHC) type I or II on the surface of an Ag presenting cell (APC). There are two major subsets of T cells, expressing different surface Ags associated with the TCR. CD8+ cytotoxic (Tc) T cells recognize Ag presented on MHC class I and can mediate killing of virus-infected or transformed cells. CD4+ helper (Th) T cells, on the other hand, bind Ag presented on
MHC class II and play a central role in the induction of immune responses by providing help to B and Tc cells. Th cells can be further divided into Th1 and Th2 cells based on the cytokines that they produce. Th1 cells produce IFN-γ, IL-2, IL-12 and TNF-α and are involved in cell-mediated immunity and inflammatory responses such as delayed-type hypersensitivity (DTH) reactions. Th2 cells, on the other hand, produce IL-4, 5, 6, 9, 10 and 13 and support Ab production.

Whereas all nucleated cells express MHC class I, only certain dedicated APCs, including dendritic cells (DCs), macrophages, B cells and thymic epithelial cells, constitutively express MHC class II. DCs have been in focus in recent years due their ability to function as efficient APCs in triggering T cells (reviewed in [10]). It is thought that the Ag is taken up in the periphery by DCs which then migrate into the secondary lymphoid tissue (spleens and lymphnodes) where they present these Ags and prime specific T cells. In the secondary lymphoid tissue, several cells are present including B and T cells, DCs, macrophages and follicular dendritic cells (FDCs). Upon an immune challenge, activated B and T cells in primary follicles in secondary lymphoid tissue, form structures called germinal centers (GCs) (reviewed in [121]). It is most likely within GCs that B and T cells interact to form the functional units of immunological memory, in the form of "memory" B and T cells.

There are five Ab classes produced (IgD, IgM, IgG, IgA and IgE). An Ab is composed of two identical Ag-binding parts (F(ab') 2) and one part (Fc) that mediates different effector functions due to the ability to activate complement or bind to certain receptors (FcRs). In the primary response, mainly IgM with low affinity for the Ag is produced. The secondary response, which is more rapid than the primary response, is characterized by production of IgG, IgE and IgA Abs. These have higher affinity for the Ag and their Fc-part has effector functions that differ from those of IgM.

To avoid damage to the host, the immune system must be carefully regulated. This is important both for optimal function and to avoid pathological conditions like autoimmunity or allergy. The mechanisms by which the immune system is regulated are still largely unknown. During an Ab response to a foreign Ag, the newly formed specific Abs are able to regulate their own production. This phenomenon is known as Ab-mediated feedback regulation (reviewed in [86]). Experimentally, this can be shown in vivo by immunizing mice with specific Abs together with an Ag. The outcome of such an immunization procedure is either an up-regulation (feedback enhancement) or down-regulation (feedback suppression) of the specific Ab response. Specific IgM, IgG and IgE Abs have all been reported to have both a suppressive and an enhancing capacity. Whether the Ab response is enhanced or suppressed depends on the nature and dose of the Ag as well as the class and dose of the Ab used. This thesis will focus on the feedback regulation of specific Ab responses by Abs, complement and FcRs.
Feedback regulation of antibody responses

Feedback suppression by antibodies

The ability of IgG to suppress the primary Ab response to particulate Ags such as sheep red blood cells (SRBC) is probably the most well-known type of feedback regulation. Administration of specific IgG Abs together with SRBC suppresses over 95% of the the specific primary Ab response. This astonishing effect of IgG is very successfully used in the clinic to prevent Rh- women from becoming immunized against fetal Rh+ erythrocytes via trans-placental hemorrhage during the pregnancy. The implementation of Rh-prophylaxis in the clinic has dramatically reduced the frequency of hemolytic disease in newborn children (reviewed in [21]) and is a good example of a "success story" in transferring immunological knowledge from the laboratory to clinical practice.

The mechanisms of IgG-mediated feedback suppression has long been a subject for speculation and several models have been suggested. One hypothesis is the "masking of epitopes" in which the injected IgG hides the antigenic epitopes and thereby prevents the B cell from interacting with the Ag. The second proposed mechanism involves Fc-FcR interactions. IgG-coated particles could bind to FcRs on host phagocytic cells and thereby be eliminated by phagocytosis before inducing activation of B cells. The third hypothesis involves specific inhibition of B cells by crosslinking of BCR and the inhibitory low affinity receptor for IgG (FcγRIIB). However, it was recently shown that IgG could suppress a primary Ab response in mice deficient for FcγRIIB (FcγRIIB−/− mice), FcγRI+FcγRIII (mice lacking the common Fcγ chain, FcγRIγ−/− mice), FcγRI+FcγRIIB+FcγRIII (FcγRIIB−/−×FcRγ−/− mice) [99]. The effective suppression seen in the absence of all known FcγRs suggests that "masking of epitopes" may explain IgG-mediated suppression in vivo. This hypothesis is strengthened by the fact that IgM [23, 138, 196], F(ab')2, [28, 99, 181] all IgG subclasses, including IgG3 [23, 92] that is unable to bind to FcγRIIB, and IgE [99] are all reported to be suppressive.

Feedback enhancement by IgG

IgG can also mediate feedback enhancement of Ab responses. The ability of IgG to enhance Ab responses was brought to attention when, in early studies, mice immunized with Ag in complex with antisera mounted stronger immune responses than mice injected with Ag alone [172, 173]. Mice immunized with specific IgG in complex with a soluble Ag, such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH), have augmented Ab responses compared to mice immunized with the Ag alone. The magnitude of the enhanced responses is impressive. For example, anti-KLH Ab responses to IgG/KLH complexes can be over 1000- fold higher than to KLH alone.
The use of IgG1, IgG2a and IgG2b monoclonal Abs (mAbs) revealed that primary IgG [37, 48, 71, 201, 202] and IgM [201] responses can be enhanced and sustained for several months [37, 203]. Monoclonal IgG also induces enhanced secondary Ab responses [37, 107]. Moreover, injection of 2,4,6-trinitrophenyl (TNP)-conjugated KLH in complex with TNP-specific IgG induces increased numbers of Ab-producing B cells 5-6 days after challenge [48, 201]. Using immune complexes (ICs) formed by Abs in hyperimmune antiserum and Ag, potent priming for memory responses have been observed [78, 106, 107, 111, 184]. Moreover, injection with IgG/Ag complexes results in increased germinal center formation [111, 115] and increased somatic hypermutation in GC B cells [140]. Also affinity maturation of Abs in the secondary response is enhanced [111, 140]. The efficiency of IgG to enhance does not correlate with its affinity [37, 202]. An Ab specific for one epitope of an Ag can enhance responses to all epitopes on that Ag i.e. the enhancement is non-epitope specific [37, 71, 201-203].

Even though feedback enhancement by IgG has been studied for very long, the mechanism is poorly understood. Theoretically, IgG when bound to an Ag could form large complexes that would be more immunogenic and thereby trigger a more potent Ab response than a small Ag alone. This, however, is not a satisfactory explanation since removal of IgG/Ag aggregates by ultracentrifugation, does not reduce the ability of the remaining non-aggregated IgG/Ag complexes to enhance [106]. In addition, F(ab')2 fragments are less efficient than intact IgG to enhance [106]. The Fc-part is therefore most likely needed for the enhancing function of IgG [106]. IgG can fix C1q and thereby activate the classical pathway of the complement system, implying that complement may be involved in the mechanism. Mice depleted of complement by treatment with cobra venom factor (CVF) show impaired trapping of aggregated IgG [142] or IgG/Ag complexes in spleen [106, 108] and their capacity to prime B cells is abolished [49, 106]. In addition, both trapping of radiolabeled IgG in the spleen and the ability of monoclonal IgG to activate complement in vitro correlated with feedback enhancement [37, 107, 202]. On the other hand, mutated IgG2a and monoclonal IgG1, both unable to activate complement, can enhance Ab responses efficiently [203]. Findings that are put forward to suggest that complement is needed for IgG-mediated enhancement may alternatively reflect the fact that complement deficient mice have generally poor Ab responses to low doses of Ag [108]. The lack of enhancement in CVF-treated mice is therefore not necessarily IgG-related. Other mechanisms, such as involvement of FcγRs, should also be considered in seeking an explanation to the enhancing effect of IgG.

**Feedback enhancement by IgM**

IgM is found in the circulation in the form of penta- or hexameric molecules and is a potent activator of the complement system via the classical pathway. One IgM molecule
is sufficient to bind C1q and to mediate lysis of an erythrocyte [20]. In order to activate complement, IgM must change its conformation [11, 100]. The ability of IgM to enhance Ab responses is a well-studied phenomenon. IgM enhances primary IgM [35, 85, 87, 196] and IgG responses [87, 93], as well as memory cell [93] and Th cell induction [79]. T cells are required since IgM-mediated enhancement does not function in nude mice [35, 38, 117]. As early as 3 days after injections with IgM/Ag complexes an enhanced IgM response can be detected as IgM-producing cells in a plaque forming cell (PFC) assay [87]. The Ab response is sustained as enhanced IgG titers for at least 3 months [88]. In general, IgM enhances Ab responses only to particulate Ags such as SRBC [35, 85, 87, 196] and malaria parasites [79] although enhancement of responses to a soluble Ag, KLH, has also been observed [37, 48]. Administration of low Ag doses is required for enhancement to take place [117, 149, 196], probably due to the fact that particulate Ags are rather "immunogenic" by themselves. In order for IgM to enhance, it needs to be able to activate complement. A mutated monoclonal IgM, unable to activate complement, is unable to enhance and mice depleted of C3 by treatment with CVF have impaired Ab responses to IgM/Ag complexes [89].

**Feedback enhancement by IgE**

Feedback enhancement mediated by IgE is a relatively recent discovery. The first indication that IgE could have the capacity to feedback regulate an Ab response was found in vitro. IgE, due to interaction with the low affinity receptor for IgE (FcεRII, CD23), was able to focus Ag on the surface of B cells and present the Ag for T cells [102, 148]. In 1993 it was shown for the first time that specific IgE in complex with Ag could induce a strong specific Ab response in vivo [90]. Moreover, IgE could effectively enhance the number of specific B cells [200], the primary IgM-, IgG- and IgE-responses and induction of memory [72]. IgE-mediated enhancement functions with a variety of soluble Ags such as BSA, ovalbumin (OVA) and tetanus toxoid, but not with KLH or SRBC [72]. Feedback enhancement by IgE in vivo is mediated via CD23, since blocking of the receptor with anti-CD23 mAb totally inhibited the effect [72, 90]. The total dependency of CD23 for IgE-mediated enhancement was later confirmed in CD23-deficient mice [58, 75]. The effect is most likely mediated by B cells, since transfer of splenic or bone marrow cells from wild-type mice to CD23+/ mice restores IgE enhancement [75].
Complement factors can be produced by a variety of cells but the main production takes place in the liver by hepatocytes [4]. Activation of the complement cascade results in cleavage of complement proteins that leads to the formation (in the terminal pathway) of the membrane attack complex (MAC) on the plasma membrane of target cells by self-assembly of the complement molecules named C5b, C6, C7, C8 and C9. These molecules form a channel-like structure where C5b/C6/C7/C8 form the cell surface anchor to which 10-15 C9 molecules bind and form a channel in the cell membrane (reviewed in [137]) (Figure 1). Not only does activation lead to the terminal pathway but also to formation of split products (C3a, C4a and C5a) with proinflammatory properties (reviewed in [57]) or that can function as opsonins (C3b, iC3b, C3d, C4b, C4d). Three alternative pathways, the classical, alternative and mannan-binding lectin (MBL), can initiate complement activation (Figure 1). The classical pathway as discussed above, is initiated by IgM or IgG Abs which, when bound to an Ag acquire the ability to fix C1 (composed of C1q, C1s and C1r), a complex that in turn cleaves C4. The MBL pathway is initiated when MBL binds to mannose on surfaces of pathogens and thereby recruits MASP, which then cleaves C4. The alternative pathway is activated in the absence of Abs by the direct binding of C3 to the surface of pathogens. The key event in the complement activation cascade is when C3a is cleaved from C3 by a C3 convertase (C4bC2a) initially formed from the classical/MBL pathways or C3bBpP in the alternative pathway (Figure 1). The remaining C3b fragment can activate the terminal pathway by associating with C3 convertase to form C5 convertase, the enzyme that cleaves C5 into C5b and C5a, where C5a acts as a chemoattractant provoking inflammation and C5b functions as an anchor to form the MAC. C3b can also be further processed into iC3b, C3c, C3d(g) and C3d where the covalent attachment of C3d to ICs is important for the induction of Ab responses. The importance of C3 in mounting a normal humoral immune response was first illustrated when mice depleted of C3 by treatment with CVF severely diminished Ab responses [145]. Further, mice with genetic disruption of C3 [199] or C4 [55] have impaired Ab responses to T cell-dependent Ags. In contrast, factor B-deficient mice [126] have normal Ab responses suggesting an important role for the classical pathway in the regulation of Ab responses. Complement may interact with complement receptors (CRs) and thereby influence Ab responses.
Figure 1. Activation of the complement system.
CR1 (CD35) and CR2 (CD35) are glycoproteins composed of a series of amino acid repeating elements called short consensus repeats (SCRs) (Figure 2). Whereas human CR1 and CR2 are encoded by separate genes, murine CR1 and CR2 are splice variants encoded by the same gene on chromosome 1 \[114, 134\]. Murine CR2 contains 15 SCRs and CR1 has 6 additional SCRs. Murine CR1 and CR2 (CR1/2) are homologous to human CR1 and CR2 (reviewed in \[94\]). Murine CR1/2 are expressed on FDCs, B cells \[53, 105\] and peritoneal mast cells \[65, 150\]. Factors C3b, iC3b, C3d, C3d(g) and C4b covalently bound either to ICs or to an Ag can interact with and function via CRs (reviewed in \[26\]). The binding of complement factors to CR2 is crucial for efficient Ab responses. Blocking of CR1/2, but not CR1 alone, resulted in impaired Ab responses to the T cell-dependent Ags, horse red blood cells (HRBC) and KLH \[91\]. Injection of soluble human CR2, competing with cell-bound CR2 for ligand, resulted in an impaired Ab response to T cell-dependent Ags \[82\]. Ab responses to T cell-dependent Ags are largely reconstituted in CR1/2\(^{-}\) mice with transgenic expression of human CR2 \[124\]. The importance of CR1/2 for mounting a normal Ab response was confirmed by two groups studying CR1/2\(^{-}\) mice \[2, 133\]. By the use of chimeric mice, an important role of CR1/2 on B cells in mounting an Ab response and in the survival of B cells in the GCs was suggested \[2, 39, 54\]. However, reciprocal bone marrow transfers showed that CR1/2 expression on both B cells and FDCs is essential for IC trapping and long-term Ab responses to SRBC and KLH \[50\]. A number of observations therefore suggest that the expression of CR2 on B cells is crucial for the induction of Ab responses. The importance of C3d for an Ab response was illustrated by the finding that a C3d-hen egg lysozyme (HEL) fusion protein was 1000-10,000 fold more immunogenic than HEL alone \[44\]. The effect could be inhibited with blocking of the C3d interaction site on CR1/2 by the 7G6 mAb (Figure 2) \[44\].

**Figure 2.** Murine complement receptor 1 and 2. Binding sites on CR1/2 for C3-fragments C3d and C3b are indicated as well as the binding site for the C3d-blocking mAb 7G6 \[104\], which inhibits Ab production. CR1 has one extra binding site for C3b and therefore has a higher affinity for C3b than does CR2.
On B cells, CR2 is associated with CD19 and TAPA-1 to form a signaling complex (CR2/CD19/TAPA-1-complex) (reviewed in [52, 183]). Co-ligation of the CR2/CD19/TAPA-1-complex with the BCR lowers the threshold for the amount of Ag needed for B cell activation in vitro by a factor of 10- to 100-fold [27]. Moreover, mice deficient for CD19 have an impairment of Ab responses to T-dependent Ags similar to that detected in CR1/2−/− mice [161]. Co-crosslinking of the CR2/CD19/TAPA-1-complex with BCR probably also leads to efficient signaling and lowers the threshold for B cell activation in vivo, thus explaining the role of CR2 in Ab production.
Fc receptors

FcRs are found on essentially all cells in the immune system. Crosslinking of these receptors triggers a wide array of cellular responses such as phagocytosis, Ab-dependent cell-mediated cytotoxicity (ADCC) and inflammation (reviewed in [63, 95, 157]). With the exception of FcεRII (CD23), which belongs to the animal lectin family, all known FcRs belong to the Ig-superfamily. The three receptors for IgG (FcγRI, CD64; FcγRII, CD32 and FcγRIII, CD16), the high affinity receptor for IgE (FcεRI), the receptor for IgA (FcαRI) and the newly described receptor for IgA and IgM (Fcα/µRI) [166] are involved in mediating phagocytosis and other effector functions during immune responses. Another group is "transporter receptors" where the neonatal FcR (FcRn) [22] preserves the homeostasis of IgG and is responsible for transport of maternal IgG across placenta to the fetus. The poly-Ig receptor is capable of binding and transporting IgA and IgM across epithelial layers.

Most FcRs are expressed as multimeric complexes composed of one Ab-binding FcRα-chain associated with FcRβ and/or FcRγ-chains responsible for signaling. In the cytoplasmic tail of FcRγ and FcRβ there are conserved immunoreceptor tyrosine-based activation motifs (ITAMs), found also in the Igα/Igβ-chains of the BCR and CD3 of the TCR signaling-complexes. Signaling starts upon receptor crosslinking, leading phosphorylation of the ITAMs by protein tyrosine kinases, followed by cell activation. Activation via ITAM can be negatively regulated by receptors having instead, immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail. When ITIM receptors are co-crosslinked with ITAM receptors, inhibitory signaling events are initiated. The family of ITIM-containing receptors, to which FcγRIIB belongs, is growing, implying that wherever there is an "on", there is also an "off" switch to modulate signaling.

FcγRI (CD64)

Structure and cellular expression
Murine FcγRI is a 70 kDa transmembrane glycoprotein [152] encoded by a single gene located on chromosome 3 [3, 165]. FcγRI contains three extracellular Ig-like domains [3, 165], in contrast to FcγRII and III that have only two (Figure 3). Domain 1 is responsible for structural stabilization and domain 2 is the ligand-binding domain. Domain 3 is responsible for the high affinity ligand-affinity [96, 97]. Domains 1 and 2 are functionally homologous in FcγRI and FcγRII/III. The functional receptor complex is composed of a ligand-binding FcRα-chain that is (as well as FcγRIII [153, 159], FcεRI [15], PIR-A [110] and TCR-CD3) non-covalently associated with a dimer of the
**Figure 3.** Murine Fc receptors for IgG and IgE. The upper panel illustrates IgG receptors and the lower, the IgE receptors. The FcRα represents the Ab-binding chain and FcRγ and FcRβ are the associated signaling chains. Closed boxes illustrate ITAM motifs and open boxes, ITIM motifs. The closed circle indicates the motif responsible for inhibiting phagocytosis by FcγRIIB1.
ITAM-containing signaling molecule, FcRγ (Figure 3). The association with FcRγ also increases the binding affinity of the receptor for IgG [130].

Due to the lack of mAbs specific for murine FcγRI, the fact that the receptor binds the Fc-portion of IgG Abs and that the co-expression of FcγRI with other receptors with the ability to bind IgG, the cellular expression of FcγRI has not been extensively studied. However, constitutive expression of FcγRI is found on murine macrophages and monocytes [190, 191] and probably on DCs [160]. In humans, constitutive expression of FcγRI is found on macrophages, monocytes and DCs and can be induced on neutrophils and eosinophils (reviewed in [63]).

**Function**
The capacity of murine FcγRI to bind monomeric IgG2a with high affinity [97, 165, 190] makes FcγRI functionally unique since FcγRII and FcγRIII both bind IgG with low affinity. Subclasses other than IgG2a have been thought not to bind FcγRI with high affinity. However, IgG3 was recently shown to bind FcγRI, although with a 5-fold lower affinity than IgG2a [59]. Further, IgG2b, but not IgG1, binds FcγRI [56]. The removal from FcγRI of domain 3 results in the capacity to bind IgG1, IgG2a and IgG2b in the form of ICs with affinity as low as that of FcγRII and III [97]. Moreover, the non obese diabetic mouse expresses a mutated variant of FcγRI where the cytoplasmic tail is absent. Interestingly, this mutated form of FcγRI binds IgG2b as well as IgG2a and IgG3 with high affinity [60, 62]. Altogether these observations suggest that under certain conditions, FcγRI has the capacity to interact with all IgG subclasses.

The precise function of FcγRI in vivo is not defined. This is mainly due to lack of specific Abs towards FcγRI and very few studies on FcγRI−/− mice, which were produced only very recently. However, FcRγ−/− mice have been available since 1994 [178] and some clue to the in vivo function of FcγRI can be given. FcRγ is not essential for expression of murine FcγRI in vitro [165]. On the other hand, expression of murine [178] as well as human FcγRI [194] in vivo, is perturbed in FcRγ−/− mice. In FcRγ−/− mice the expression of FcγRIII on NK cells, macrophages and mast cells, of FcγRI on macrophages and of FcεRI on mast cells is absent [178]. Cells from FcRγ−/− mice are unable to phagocytose or bind IgG/SRBC complexes, suggesting a crucial role of FcRγ in either surface expression of FcγRI or ligand binding. Targeting of Ag by Ag-anti-FcγR conjugates to FcγRI, FcγRII or FcγRIII on human monocytes in vitro resulted in enhanced Ag presentation, although Ag presentation was most efficient by targeting to FcγRI [66]. Isolated human DCs, targeted with an Ag-anti-FcγRI conjugate, could mediate Ag presentation and activate T cells 50- to 500- fold more efficiently than DCs targeted with Ag alone [51]. Additionally, DCs were 10-fold more potent in presenting Ag and activating T cells than were macrophages [51]. Moreover, mice with transgenic FcγRI expressed selectively on myeloid cells have enhanced Ab responses upon
targeting of Ag-mAb conjugates to FcγRI in vivo [83]. FcRγ–/– mice have impaired DTH reaction, i.e. less efficient Th1 priming, due to dysfunctional Ag presentation [77]. In addition to the function of FcγRI in Ag presentation, macrophages from FcRγ–/–, but not from FcγRIIB–/– or FcγRIII–/– mice, fail to produce IL-10 after stimulation with ICs. IL-10 down-regulates pro-inflammatory responses, suggesting FcγRI also mediates immunostimulatory functions [174]. Protection from IC-induced peritonitis is FcγRI-dependent [84]. However, FcγRI seems to be less important for development of IC-induced experimental autoimmune hemolytic anemia since FcRγ–/– mice were completely protected and FcγRIII–/– mice partially protected (to IgG1 and IgG2a ICs), while FcγRI–/– mice were not protected from developing anemia [56].

**FcγRII (CD32)**

**Structure and cellular expression**

The structure of FcγRII differs from FcγRI and III in that FcγRII is a single chain receptor composed of two extra-cellular Ig-like domains and a cytoplasmic tail [159] (Figure 3). Three different genes encode the human variants (A, B and C) which in turn give rise to six different splice variants. The A and C forms have ITAMs in the cytoplasmic tail whereas FcγRIIB has an ITIM. Recently, the three dimensional structures were described for the human A [127] and B [169] forms. The two Ig-like domains were found to be bent at an angle of approximately 70 degrees, exposing the ligand binding site in domain 2 [127, 169]. In mice, only the inhibitory FcγRIIB is expressed. From the single gene encoding FcγRIIB, located on chromosome 1, four different splice variants are formed that differ only in the cytoplasmic tail. The isoforms are termed B1, B1’, B2 [116, 118, 151, 159] and B3, the last named being a soluble variant of B2 expressed in macrophages [182].

All hematopoetic cells, except erythrocytes and NK cells, express FcγRIIB, (reviewed in [40, 63, 95, 156]). B1 and B1’ are expressed mainly on B cells and are the only FcγRs on these cells [116, 159]. B2 is mainly expressed on macrophages [159].

**Function**

FcγRIIB binds IgG1, IgG2a and IgG2b with low affinity whereas IgG3 does not bind at all [198] (also reviewed in [63, 95]). IgE binds FcγRIIB2 with low affinity [180].

Activation signals initiated by ITAM-receptors such as BCR or FcRs associated with FcRγ-chain can be attenuated by ITIM containing receptors [41] (also reviewed in [40, 158]). Cell activation is terminated upon co-ligation with FcγRIIB and this can lead to apoptosis in B cells [7, 144] and eosinophils [43]. Additionally, effects on B cell proliferation [146, 167], signaling [14, 31, 135, 205], Ig-secretion [146, 147], and Ag
presentation [5, 129, 131, 192] have been observed. Upon co-ligation of FcγRIIB with FcεRI on mast cells, degranulation and cytokine production is inhibited [42], suggesting that FcγRIIB is able to modulate activation mediated by FcεRI as well.

The phenotype of mice deficient for FcγRIIB (FcγRIIB−/−) substantiated the findings of the function displayed in vitro [179]. The Ab responses in these mice are elevated to T-dependent and T-independent Ags and mast cells from FcγRIIB−/− mice are more sensitive to degranulation, resulting in increased passive cutaneous anaphylaxis reactions [179, 187]. Moreover, FcγRIIB−/− mice are more susceptible for developing IC-related autoimmune diseases such as collagen-induced arthritis [109, 211], Goodpasture's syndrome [139], lupus [18] and IC-induced alveolitis [33]. In addition, a more potent anti-tumor function via ADCC with a tumor specific Ab is seen in FcγRIIB−/− mice [34].

FcγRIII (CD16)

Structure and cellular expression

The FcRα-chain of murine FcγRIII displays 95% amino acid homology to murine FcγRII and is also recognized by the same mAb, 2.4G2 [190]. Moreover, FcγRIII also binds to ICs containing IgG1, IgG2a or IgG2b [189]. It was therefore initially classified as a FcγRII variant. However, due to the similarities to human FcγRIIIA in structure, function and cellular distribution it is now classified as FcγRIII. In mice, only one form of FcγRIII is expressed but in humans there are two, FcγRIIIA and FcγRIIIB (reviewed in [63, 95]). To become functionally active, FcγRIII has to associate with FcRγ; this is necessary for expression [113, 153], signaling [19, 206] and internalization [6]. On mast cells, FcγRIII is associated with both FcRγ and FcRβ (Figure 3) where the latter acts as an amplifier of FcγRIII responses by enhancing FcRγ-mediated signaling [46, 112].

Murine FcγRIII is expressed on macrophages, mast cells and possibly on DCs [12, 101, 159, 160, 198]. It is the only FcγR expressed on both murine and human NK cells (reviewed in [63, 95]).

Function

The effector function of FcγRIII has been studied in FcRγ−/− mice (lacking both FcγRI and FcγRIII) and in mice selectively knocked out for FcγRIII (FcγRIII−/−). FcRγ−/− mice have dysfunctional cytotoxicity reactions mediated by NK cells and macrophages [178] and impaired rejection of melanoma and lymphoma, suggesting an important role for FcγRI and/or FcγRIII in ADCC [32, 197]. FcγRIII−/− mice displayed impaired NK cell-mediated ADCC, no IgG-mediated passive cutaneous anaphylaxis reaction and impaired IgG-dependent Arthus reaction [80]. IgG-mediated degranulation of mast cells
from FcγRIII−/− mice was abrogated and macrophages could not mediate phagocytosis of IgG1/Ag complexes [80, 81]. In FcγRI−/− mice, IgG1-mediated anaphylaxis [45, 132] seems to be mediated by FcγRIII. Moreover, a role for FcγRIII in development of IC-related autoimmune disease is suggested [56, 128]. The severity and induction of autoimmune hemolytic anemia is dependent on the IgG subclasses administrated, where IgG2a and IgG1 seem to be most pathogenic and dependent on FcγRIII expression [56, 128]. In addition, FcγRI−/− mice are protected from the development of experimental autoimmune arthritis induced by collagen, suggesting an additional role for FcγRI and/or FcγRIII in the development of arthritis [109].

**FcεRII (CD23)**

Murine FcεRII (CD23) (Figure 3) is constitutively expressed on B cells [64, 154] and FDCs [122] whereas human CD23 is expressed on B cells, T cells, FDCs, eosinophils, platelets, macrophages and NK cells (reviewed in [36]). The receptor is composed of three single chains forming a coiled structure [13] (Figure 3). IgE/Ag complexes that bind CD23 on B cells induce receptor-mediated endocytosis [70] and mediate Ag-presentation for T cells in vitro [102, 148]. In contrast, co-crosslinking of CD23 with the BCR induces apoptosis of splenic B cells [25], and prevents B cells from proliferating and differentiating [24, 120]. CD23-deficient mice (CD23−/− mice) have been generated by three groups [58, 171, 209]. Feedback enhancement by IgE/Ag complexes, previously found to be mediated via CD23 [72, 90], is impaired in CD23−/− mice [58, 75]. The IgE production is increased [209], suggesting a role for CD23 in regulating the serum level of IgE. Mice with a transgenic overexpression of CD23 have an impaired IgG1 and IgE response [143, 185].
The present investigation

Aims

The purpose of the present investigation was to study the biological mechanisms of Ab-mediated feedback enhancement.

Specific aims were to:

• Investigate the role of CR1/2 in IgM-, IgE-, and IgG2a-mediated enhancement (I).

• Elucidate involvement of FcγRs in feedback enhancement of Ab responses mediated by IgG1-, IgG2a- and IgG2b- ICs (II, III).

• Analyze the modulatory role of FcγRIIB in responses to IgG/Ag complexes (II, III).

• Study feedback enhancement by IgG3/Ag complexes and involvement of FcγRs and complement (IV).
The experimental model

Mice

The majority of available knock-out mice have a mixed 129/Sv×C57BL/6 genetic background and carry the H-2^b MHC haplotype. H-2^b mice have an I-A^b-linked low responsiveness to IgG and IgE in complex with soluble Ags [71, 73]. The FcR\gamma^-/- [178], Fc\gammaRIIB^-/- [179], Fc\gammaRIII^-/- [80] or CR1/2^-/- [133] mice used in the present investigation were therefore backcrossed with mice of responder strains (CBA/J (H-2^k), DBA/1 (H-2^q) or Balb/c (H-2^d)). Screening for MHC, knock-out or wild-type alleles, were done with by PCR. Mice from either the first (I, II, III), fifth (III, IV) or tenth (III) backcross-generations were used.

Methods

Antibodies

mAbs were derived from B cell hybridomas producing TNP-specific IgE (IGELb4 [162]), IgG1 (B8401H5) [37], IgG1 [141], IgG2a (C4007B4) [37], IgG2b (C1901B4) [37], (GKH-1-GORK) (a gift from Dr. G. Köhler (Max Planck Institute Freiburg, Germany)) or IgG3 (IM-F10, IM-H11) (derived in our laboratory). Polyclonal IgM anti-SRBC was purified from sera obtained from mice five days after intraperitoneal immunization with SRBC in phosphate buffered saline (PBS).

Antigens

BSA, OVA, SRBC and HRBC were used as Ags. OVA and BSA were either unconjugated or TNP-conjugated (BSA-TNP or OVA-TNP). Immune complexes were formed by incubating the TNP-specific mAb together with the TNP-conjugated Ag for 1 h at 37°C immediately before injection. As a specificity control for feedback enhancement, unconjugated Ag was added to each mixture (OVA to the BSA-TNP and BSA to the OVA-TNP).

ELISA

Mice were bled from the tails and, sera was analyzed for specific IgG by BSA-, OVA- or SRBC-specific ELISAs. 96-well microtiter plates were coated with the Ag (BSA, OVA or SRBC) and sera were serially diluted and added to the plates. The amount of specific IgG was determined using sheep anti-mouse IgG conjugated with alkaline phosphatase. OVA- or BSA-specific affinity purified polyclonal IgG was used as a standard to determine the concentration of OVA- or BSA-specific Abs.
ELISPOT
ELISPOT was used to determine the number of B-cells secreting specific IgG. Briefly, spleen cells were added to Ag-coated microtiter plates and incubated at 37°C. Specific IgG was detected using sheep anti-mouse IgG. The spots were developed using 5-bromo-4-chloro-3-indolyl phosphate and counted under a microscope.

PFC
By a direct hemolytic PFC assay the number of B-cells producing specific IgM can be detected. Briefly, spleen cell suspensions were added to a mixture of SRBC or HRBC, complement and agar. The mixture was spread on slides, incubated at 37°C and the cells producing SRBC- or HRBC-specific IgM were counted as direct plaques i.e. lysed erythrocytes.

CVF treatment
CVF treatment of mice causes transient depletion of C3 to less than 5% of normal levels. CVF works by displacing C3b in C3 convertase of the alternative pathway, forming a more stable C3 convertase (see Figure 1) with a half-life of several hours. Normal mice were injected intraperitoneal with four doses of 100 µl of 100 U/ml CVF over the course of 24 h. Levels of C3 in sera were assayed by radial immunodiffusion using a polyclonal goat anti-mouse C3 antiserum.

DTH reaction
DTH is a cell-mediated inflammatory response induced by Th1 cells [30]. To be able to test the DTH reaction, mice were primed with 10 or 100 µg of BSA emulsified in complete Freund's adjuvant (CFA) injected intradermally at the root of the tail. Challenging for DTH was done by injecting 10 µg BSA in PBS i.v. in the right ear and as a control, PBS alone was injected in the left ear. As a measurement of the DTH reaction, ear swelling was measured (with an accuracy of 0.01 mm) by subtracting the ear-thickness of the left from the right ear.

Statistical analysis
Statistical differences between the control and the experimental groups were determined by Student's $t$ test.
Results and discussion

CR1/2 in feedback enhancement of Ab responses (I)

IgM-mediated enhancement of Ab responses is known to be dependent on the activation of complement [89]. However, it is unknown if it is necessary for complement to bind CRs in the process of IgM-mediated enhancement. This is the topic of the present report.

Impaired Ab responses to IgM/Ag complexes in CR1/2−/− mice

CR1/2−/− and wild-type mice were immunized with suboptimal doses of SRBC alone or together with IgM anti-SRBC. IgM enhanced the specific Ab response, measured as IgG titers in serum by ELISA and as specific IgM-producing cells by direct PFC assay in wild-type but not in CR1/2−/− mice. In an alternative approach, wild-type mice were treated with 200 µg of anti-CR1/2 mAbs (7G6) [104] 24 h before challenge with IgM-anti SRBC and SRBC. This also resulted in the inability of IgM to enhance specific Ab responses, as measured by PFC. The enhancement was Ag-specific since no enhancement to an irrelevant Ag (HRBC) was detected. Further, we tested whether CR1/2−/− mice are responsive to SRBC under conditions when optimal Ag doses were injected alone. Increasing the dose of SRBC (from 4×10^5 to 1×10^8 per mouse) led to activation of SRBC-specific B cells both in wild-type (from 3630 to 10232 PFC) and in CR1/2−/− mice (from 170 to 1622). The increased response in CR1/2−/− mice demonstrates the ability of these mice to produce an Ab response to high dose immunizations. Feedback enhancement by IgM/Ag complexes is therefore most likely due to a selective block of the IgM/complement-dependent pathway of B cell activation rather than a complete inability of CR1/2−/− mice to respond to SRBC.

IgG2a/Ag, IgE/Ag, or Ag in adjuvant induces normal Ab responses in CR1/2−/− mice

Wild-type and CR1/2−/− mice were immunized with IgG2a/BSA-TNP or IgE/BSA-TNP complexes. Both wild-type and CR1/2−/− mice were able to respond equally well to IgG2a/Ag and IgE/Ag complexes.

Challenging wild-type and CR1/2−/− mice with various amounts of BSA in CFA or alum resulted in the production of BSA-specific IgG both in wild-type and in CR1/2−/− mice. Although the Ab response was slightly higher in wild-type mice, the difference was not significant.
Discussion (I)

These results show that CR1/2−/− mice are able to produce high Ab titers after immunization with Ag in complex with IgE or IgG2a or in adjuvant emulsions. The expression of CR1/2, therefore, is not an absolute requirement for a normal Ab response to be initiated. This is in agreement with previous data showing that CVF-treated animals respond to Ag administered in adjuvants [125, 145] and is compatible with the idea that only responses to suboptimal Ag doses are dependent on the complement system.

IgE-mediated enhancement requires expression of CD23 and even though IgE cannot activate complement, a possible link to the complement system exists, since human CD23 is a ligand for CR2 [8]. However, the ability of IgE to induce feedback enhancement in CR1/2−/− mice shows that CR2 is not required. As mentioned, the dependence of IgG-mediated enhancement on complement has been debated. Early studies showed that IgG-mediated enhancement of B cell memory was abolished in complement deficient mice [106] and that the ability of IgG to enhance correlates with the ability to activate complement [37, 202]. The ability of mutated IgG2a and normal IgG1 mAbs [203], both unable to activate the classical pathway, to enhance Ab responses suggested that the need for complement in IgG-mediated enhancement is not absolute. This conclusion is also strengthened by the fact that IgG2a/Ag complexes are able to enhance specific Ab responses in CR1/2−/− mice.

Previous studies showing that complement is needed for IgM to enhance [89] and that CR1/2 is required for a normal Ab response [2, 39, 91, 133], implied that feedback enhancement by IgM is mediated via CR1/2. That this is indeed the case is demonstrated in the present investigation. Physiological primary responses do not initially involve Ag binding at the high levels of specific IgM Abs that we have used in the present study. Rather, the Ag forms complexes with natural IgM. Recent observations support the important role for natural IgM in the induction of Ab responses. Mice lacking secretory IgM, due to gene targeting, but retaining membrane bound IgM and the ability to secrete IgG and IgA, had diminished Ab responses to T cell-dependent Ag [17, 47]. Responses could be reconstituted with administration of IgM from normal mouse serum before challenge [47]. It therefore appears likely that administration of Ag in low doses involves recognition of the Ag by natural IgM followed by activation of complement and ligation of CR1/2 that in turn would promote production of early specific IgM able to feedback enhance the Ab response further.

The exact mechanism by which CR1/2 operates in the induction of Ab responses is not fully understood. Co-ligation of IgM/Ag/complement with the CR2/CD19/TAPA-1 complex lowers the threshold for activation of Ag-specific B cells [27, 186] (Figure 4) and could be one explanation. Alternatively, CR1/2-facilitated Ag presentation to T cells by Ab/complement-opsonized Ags, seen in vitro [9, 16, 204], may be involved.
Against this hypothesis is the fact that T cell priming can occur in vivo without functional CR1/2 [74] or in the absence of C3 [55]. Regardless of which molecular mechanism(s) is involved, the data presented here reveal two important aspects of the in vivo role of CR1/2. First, expression of CR1/2 is crucial for IgM-mediated triggering of Ab responses and second, the need for CR1/2 can be circumvented when the Ag is in complex with either IgG2a or IgE, Ab types of secondary responses, or in an emulsion of adjuvant, mimicking natural inflammatory responses.

**Figure 4.** B cell activation by co-crosslinking the BCR with CR1/2 mediated by Ag/IgM/complement-complexes. The engagement of CR1/2 in IgM-mediated enhancement lowers the threshold for B cell activation.

**FcγRs in feedback enhancement of Ab responses (II, III)**

The fact that IgG can enhance Ab responses in CR1/2−/− mice supports previous findings where non-complement activating IgG2a and IgG1 could enhance Ab responses. Since simple aggregation of the IgG/Ag complexes cannot explain the enhancing capacity of IgG [106], involvement of FcγRs seemed likely. In the present reports (II, III) the involvement of FcγRs in the modulation of feedback enhancement was studied.

**Impaired IgG-mediated enhancement in FcRγ−/− mice (II)**

Wild-type and FcRγ−/− mice (lacking functional expression of FcγRI, FcγRIII and FcεRI) were immunized with BSA-TNP alone or in complex with TNP-specific IgG1, IgG2a or IgG2b mAbs. Specific IgG anti-BSA titers were measured in serum collected from mice 14, 21 and 28 days after challenge. As expected, all IgG isotypes enhanced in wild-type mice. Interestingly, IgG1- and IgG2a-mediated enhancement in FcRγ−/− mice was almost completely absent and enhancement by IgG2b was reduced at early time points. No responses towards OVA were detected in any of the experiments, confirming the specificity of enhancement.
Because FcRγ is also associated with the TCR-CD3 complex, the impaired IgG-mediated enhancement in FcRγ-/- mice could therefore reflect aberrant Th cell function in these mice rather than a FcγRI or FcγRIII effect. However, Ab responses to Ag in adjuvant is normal in FcRγ-/- mice strengthening the hypothesis that these mice have normal Ab responses [178]. To exclude the possibility that the impaired Ab responses in FcRγ-/- mice were due to impaired Th cell function, wild-type and FcRγ-/- mice were challenged with TNP-specific IgE in complex with BSA-TNP. The ability to respond to IgE/Ag complexes is solely dependent on CD23 expression [58, 72, 75, 90] and lack of response would therefore indicate defects in TCR-CD3, rather than in FcγRI or FcγRIII. Wild-type and FcRγ-/- mice responded equally well to IgE/Ag complexes, suggesting IgG-mediated enhancement, dependent on the FcRγ chain in FcγRI FcγRIII.

Normal Ab responses in FcγRIII-/- mice (II)
Since FcRγ-/- mice lack functional expression of both FcγRI and FcγRIII it is not possible, from use of this strain, to conclude whether FcγRI or FcγRIII contributes to IgG-mediated enhancement. Mice selectively lacking FcγRIII (FcγRIII-/- mice) were therefore immunized using the same experimental setup as described above. IgG of all isotypes (IgG1, IgG2a and IgG2b) were able to enhance the Ab responses in wild-type and in FcγRIII-/- mice, without major differences in magnitude of the responses.

Discussion (II)
The assumption that expression of FcγRIIB is normal in FcRγ-/- mice, together with their low responsiveness to IgG/Ag complexes implies that FcγRIIB is not capable of inducing enhancement on its own. The unaltered response in FcγRIII-/- mice implies that FcγRI alone is sufficient to enhance. It is possible be that FcγRI is the only receptor involved, although involvement of FcγRIII, by co-operation with FcγRI, cannot be excluded. However, the expression pattern of FcγRIII, together with its ability to mediate effector functions such as mast cell degranulation and ADCC, implies that FcγRIII is primarily involved in inflammatory responses rather than in mediating Ag presentation to T cells in vivo. Involvement of FcγRI in mediating enhancement of IgG/Ag complexes, on the other hand, is not surprising. Ag (conjugated with an anti-FcγRI mAb) targeted to human FcγRI is more efficiently presented to T cells in vitro [66, 119, 195]. Such conjugates also enhance Ab responses in vivo in mice transgenic for human FcγRI [83, 103]. FcγRI is expressed on DCs and macrophages which are efficient APCs and are able to efficiently present IgG/Ag to T cells in vitro [123, 163, 188], whereas DCs seem to be most efficient in presenting IgG/Ag complexes to T cells [51]. An attractive possibility is that this mechanism also operates in vivo, resulting in enhancement of Ab responses (Figure 5). Induction of Ab responses by IgG/Ag complexes via FcγRs could be of particular importance in a secondary response when specific IgG, generated from the primary response, is already present when the Ag is
encountered. The current view that IgG2a is the only ligand that binds FcγRI with significant affinity [95] argues against a common role of FcγRI to be the only receptor mediating enhancement by IgG1, IgG2a and IgG2b Abs. However, IgG2b [56] and IgG3 [59] ICs were recently shown to bind FcγRI with low affinity in vitro. Moreover, FcγRI gains the function of binding IgG1 and IgG2b when the third extracellular domain is removed [97] and unique alleles of FcγRI exist that can bind both IgG2b and IgG3 [61, 62], demonstrating that FcγRII binds isotypes other than IgG2a. Therefore, it is likely that IgG1 and IgG2b bound to Ag could interact with FcγRI. Although the unaltered response in FcRγ/- mice to IgE/Ag (II) and to Ag/CFA [178] suggest that IgG-mediated enhancement is FcγR-dependent, the actual contribution of each FcγR in IgG-mediated enhancement has to be further characterized in FcγRII/- mice as well as in (FcγRI/- × FcγRIII/-) double knock-out mice to exclude the possibility that FcγRγ/- mice have other vital defects besides lack of FcγR expression.

The small residual enhancement detected towards IgG1/Ag and IgG2b/Ag complexes in FcRγ/- mice could be due to a contribution by the complement system. Wild-type IgG2a was shown to be slightly more potent in inducing Ab responses than a mutated, non-complement activating, IgG2a Ab [202]. A co-operative link between FcγRs and complement exists since complement increases to the IgG-induced anaphylactic responses detected in FcγRIII/-/ mice [80]. The only FcγR expressed in FcRγ/- mice is FcγRIIB. Despite the reported inhibitory function of FcγRIIB both in vivo and in vitro, FcγRIIB2 expressed on macrophages can mediate endocytosis of IgG/Ag complexes. FcγRIIB2 therefore, also represents a possible explanation for the residual enhancement detected in FcRγ/- mice.

**FcγRIIB lowers the magnitude of Ab responses to IgG/Ag complexes (II, III)**

To investigate the role of FcγRIIB in feedback enhancement of Ab responses, wild-type and FcγRIIB/- mice were challenged with either BSA-TNP alone or in complex with TNP-specific IgG1, IgG2a, IgG2b or IgE. IgE/Ag complexes induced equally efficient enhancement in wild-type and FcγRIIB/- mice 21 days after immunization whereas IgG/Ag complexes induced up to 189-fold more potent Ab responses in FcγRIIB/- mice than in wild-type mice (II).

To further substantiate this finding and to investigate the role of FcγRIIB at different stages of Ab responses, wild-type and FcγRIIB/- mice were immunized with BSA-TNP alone or in complex with TNP-specific IgG2a or IgE, alternatively with SRBC alone or together with SRBC-specific IgM. Sera taken 14, 21, 28, 56 and 84 days after immunization were analyzed for specific IgG content. Specific Ab responses were enhanced by IgG2a/Ag, IgE/Ag and IgM/Ag complexes in wild-type and FcγRIIB/- mice. After challenging with IgG2a/Ag complexes, FcγRIIB/- mice produced 21- to 61-fold higher amounts of specific Abs compared to wild-type mice. Wild-type and
FcγRIIB−/− mice immunized with IgE/Ag or IgM/Ag had equally strong Ab responses during the entire test period.

The striking efficiency of IgG/Ag complexes to enhance Ab responses in FcγRIIB−/− mice prompted us to ask whether the threshold for mounting an Ab response to these complexes is lower in FcγRIIB−/− mice than in wild-type mice. The question was investigated by using decreasing doses of IgG1/Ag and IgG2a/Ag complexes. In FcγRIIB−/− mice, 2, 10, and 50 µg of IgG2a triggered an enhanced response, whereas in wild-type mice, enhancement was only seen using 10 or 50 µg IgG2a. The magnitude of the response was 6- to 36-fold higher in FcγRIIB−/− than in wild-type mice. Furthermore, although 50 µg IgG1 could not induce an enhanced response in wild-type mice, a 17-fold enhancement was observed in FcγRIIB−/− mice. In contrast, ICs containing 2, 10 or 50 µg of IgE enhanced equally in wild-type and FcγRIIB−/− mice.

Hypothetically, enhanced affinity maturation, decreased clearance of the produced IgG Abs or an increased number of specific B cells could explain the augmentation of the Ab response detected in FcγRIIB−/− mice. Therefore, the amount of B cells producing specific IgG after immunization with IgG/Ag complexes was tested using an ELISPOT assay. In FcγRIIB−/− mice, B cells producing specific IgG were found as early as day 6 (240 spots/spleen) and were sustained until day 22 (719 spots/spleen) with a peak at day 14 (3576 spots/spleen) after immunization. Wild-type mice had lower levels of specific B cells, with a peak at day 10 (435 spots/spleen) after immunization. To examine whether the elevated number of specific B cells in FcγRIIB−/− mice was also reflected in increased GC formation, spleen sections were analyzed by staining the PNA+ cells in 15 non-consecutive cryosections taken 3, 7, 10 and 14 days after immunization with IgG2a/Ag complexes. In FcγRIIB−/− mice, GCs were formed earlier than in wild-type mice with a 3-fold higher number at day 7. Later in the response, at days 10 and 14, the number of GCs in the FcγRIIB−/− mice converged with the number of GCs in wild-type mice.

Mice of the H-2b MHC haplotype are low responders to IgG/Ag complexes. Moreover, in collagen-induced arthritis, a mouse model for rheumatoid arthritis, shown to be dependent on H-2d or H-2a MHC genes (reviewed in [136]), FcγRIIB−/− mice of the H-2b haplotype become susceptible to disease [211]. However, no enhancement of Ab responses to IgG/Ag complexes was detected in FcγRIIB−/− mice of the H-2b haplotype, demonstrating that lack of FcγRIIB could not reverse the low responsiveness.

Due to the enhanced Ab production in FcγRIIB−/− mice, the ability to mount DTH reactions could also be affected. FcγRIIB−/− and wild-type mice were primed with 10 µg or 100 µg BSA in emulsified CFA. One week later, mice were challenged i.v. in the ear with BSA and DTH reactions were analyzed by measuring the swelling of the ear 24, 48, 72 and 120 h after challenge. Additionally, serum levels of IgG were measured 14
days after primary injections. Despite the 3.6-fold higher Ab response detected in FcγRIIB−/− mice receiving 100 µg of Ag, there were no differences between the two strains in their ability to develop a DTH reaction.

Discussion (II, III)
Previous reports have shown that FcγRIIB−/− mice have a 5-fold enhanced Ab production to KLH administered in adjuvants or to SRBC, compared to wild-type mice. We have shown that this difference is even more pronounced when preformed complexes of IgG/Ag are used: as much as a 189-fold higher Ab response was detected in FcγRIIB−/− mice than in wild-type mice.

The exact molecular mechanism behind the inhibition by FcγRIIB on enhancement induced by IgG/Ag complexes is not fully understood. IgG/Ag complexes administrated in vivo are probably targeted to FcγRI present on APCs. One possibility is that FcγRI and FcγRIIB are co-crosslinked and that Ag presentation is thereby inhibited. In the absence of FcγRIIB, IgG/Ag can be more efficiently taken up and presented by APCs, leading to more primed Th cells that in turn can give co-stimulatory help to more B cells, a process that ultimately leads to enhanced Ab production (Figure 5). The importance of FcγRs in DTH reactions was recently suggested due to the observation that FcγR−/− mice had reduced ability to present Ag and to mount a DTH response [77]. Therefore, if FcγRIIB-mediated inhibition of FcγR-mediated Ag uptake and presentation were the explanation for IgG-mediated enhancement of Ab responses, this would presumably result in an enhanced ability to induce a DTH reaction in FcγRIIB−/− mice. Since this was not the case (III), our results indicate that inhibition by FcγRIIB of FcγRI-mediated Ag presentation is an unlikely explanation for the enhanced Ab responses observed in FcγRIIB−/− mice. An alternative possibility is that IgG/Ag complexes inhibit specific B cells by co-crosslinking the BCR with FcγRIIB, a mechanism demonstrated to exist in vitro [5, 131, 135, 192]. This could explain the low threshold for inducing an Ab response to IgG/Ag complexes in FcγRIIB−/− (III).

FcγRIIB−/− mice on the C57BL/6 background have recently been shown to spontaneously develop the IC-related autoimmune disease, lupus [18]. In a model, irradiated B cell-deficient mice (RAG−/− or IgH−/−) developed disease when they received bone marrow from FcγRIIB−/− mice. In contrast, they were protected from disease after receiving bone marrow cells from wild-type mice [18]. Expression of FcγRIIB on the surface of B cells, and not on macrophages, was important to protect the mice from disease [18]. These findings, along with our results, support the hypothesis that FcγRIIB expression on B cells, and not on APCs, is the most important factor in modulating Ab responses.
Immunizing with ICs composed of IgE or IgM Abs does not alter or sustain the Ab responses in FcγRIIB⁻/⁻ mice and the threshold for mounting an Ab response to IgE/Ag complexes is equal in both strains. This suggests that the inhibition by FcγRIIB of Ab responses to the injected IgG/Ag complexes appears early in the response. Such a mechanism could operate as a "safety-valve" in the secondary response where preformed IgG ICs could prevent too many new B cells from becoming activated.

**Figure 5** Positive and negative modulation of IgG-mediated enhancement via FcγRs. Open symbols indicate the ITIM-containing receptor, FcγRIIB and filled symbols indicate the ITAM-containing receptor FcγRI. To the left of the dotted line, a possible mechanism by which FcγRI can mediate more enhanced uptake of IgG/Ag complexes that are presented for T cells is shown. To the right of the dotted line, the possible mechanisms by which FcγRIIB can inhibit Ab responses are indicated.
Feedback enhancement by IgG3/Ag complexes (IV)

Although IgG1, IgG2a and IgG2b can enhance specific Ab responses no reports about the capacity of IgG3 to enhance exist. The early appearance of IgG3 in Ab responses makes the subclass interesting in terms of feedback enhancement, since early production of specific IgG3 could lead to production of other subclasses later in the response. We generated two different TNP-specific monoclonal IgG3 Abs. BSA-TNP alone or in complex with TNP-specific IgG3, was passively administered i.v. to mice. The levels of BSA-specific serum IgG were analyzed fourteen days after injection. Indeed, both IgG3 mAbs were able to enhance specific Ab responses 12.6- to 1344-fold. The magnitude depended on the mAb and mouse strain used.

The ability of IgG3 to bind FcγRI [59] and the fact that the other isotypes of IgG most probably enhanced via FcγRI made us investigate whether IgG3 could enhance in FcγR1-/- mice. If FcγRI were the sole receptor for IgG3 it is logical to assume that enhancement would be impaired in these animals. Surprisingly, this was not the case since wild-type and FcγR1-/- mice, bled 14, 21, 28 and 46 days after passive administration of 2, 10 or 50 µg of IgG3/Ag complexes, enhanced specific Ab responses up to 245-fold. Even more surprising was that the Ab response was higher in FcγR1-/- than in wild-type mice. In a parallel experiment, IgG2a/Ag complexes enhanced in wild-type but not in FcγR1-/- mice, as expected from previous studies (II).

IgG3 is the only IgG subclass unable to bind FcγRIIB. As mentioned (II, III), injection of ICs composed of IgG1, IgG2a or IgG2b and Ag induce a more potent Ab responses in FcγR1IB-/- mice compared to in wild-type mice. In contrast, the magnitude of the Ab response is the same in the two strains after administration of IgG3/Ag.

The efficient enhancement mediated by IgG3 in the absence of FcγRI or FcγRIIB prompted us to investigate if complement was involved. Complement has been reported to enhance the uptake of IgG3-coated SRBC by murine peritoneal macrophages [164]. Using heat-inactivated serum (to inactivate complement), IgG3 was still able to mediate Ag uptake by mouse macrophages deficient in both FcγRI and FcγRIIB in vitro [210]. We wanted to test the complement dependence of IgG3-mediated enhancement. Wild-type mice were depleted of C3 with CVF before immunizing with IgG3/Ag complexes. The Ab responses were enhanced in both C3-depleted and sham-treated mice, suggesting that complement activation may not be needed for IgG3-mediated enhancement.

Discussion (IV)

Enhancement in FcγR1-/- mice was unexpected since it was recently reported that IgG3/Ag complexes could bind FcγRI in vitro. Other studies have, however, indicated
that IgG3 can operate in a FcRγ-independent way. When the intracellular fungus, Cryptococcus neoformans, is opsonized in vitro with IgG1, IgG2a, or IgG2b, it is phagocytosed by macrophages isolated from wild-type, but not FcRγ−/− mice. In contrast, IgG3-opsonized Cryptococcus neoformans are phagocytosed by macrophages from both wild-type and FcRγ−/− mice. Moreover, IgG3-opsonized SRBC are able to bind macrophages from FcRγ−/− × FcγRIIB−/− double knock-out mice, suggesting that IgG3-mediated phagocytosis operates independently of known FcγRs [210]. Not only did IgG3 enhance in FcRγ−/− mice, the magnitude of the enhancement was generally higher than in wild-type mice. One explanation for this could be that a FcR for IgG3, present on APCs, could mediate endocytosis of IgG3/Ag complexes leading to efficient priming of CD4+ T cells. In the absence of FcγRI (as in the case of FcRγ−/− mice) more IgG/Ag complexes would be available for this Ag presentation pathway. The fact that enhancement by IgG3/Ag complexes functions in FcRγ−/− as well as in FcγRIIB−/− mice implies that no known FcγR is involved in the mechanism. That an undiscovered receptor for IgG3 might exist is therefore a possibility.

The mechanism by which IgG3 enhances is not clear. It is known that at least two IgG molecules in close vicinity are needed in order to fix C1q and activate the complement cascade. IgG3 has the capacity to spontaneously self-associate, via Fc-Fc interaction which leads to precipitation at low temperature [1, 76, 170]. These self-associative properties are important for the capacity to bind Ags [67-69] and may also facilitate complement activation. Complement could therefore be a feasible explanation for IgG3-mediated enhancement. However, the finding that CVF-treated mice respond equally well to IgG3/Ag complexes as wild-type mice, does not support the idea that complement is involved. On the other hand, to completely rule out involvement of complement, further experiments in mice genetically deficient for C3 are needed. An alternative possibility to explain IgG3-mediated enhancement is that IgG3 can self-associate and thereby form larger, more immunogenic, ICs.
Summary

In the following tables (Table 1 and 2) the results from previous studies as well as the studies in the present investigation are summarized.

**Table 1.** Ab responses in mice to soluble Ags administered in adjuvants or particulate Ags administered alone in PBS.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>C3 depleted</th>
<th>CR1/2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeRγ&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeγRIII&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeγRIIB&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA/Ag&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>NT&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Enhanced</td>
</tr>
<tr>
<td>[125]</td>
<td>([I, 208])</td>
<td>[77, 207]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alum/Ag&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NT</td>
<td>Normal</td>
<td>Normal</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>([I, 29, 208])</td>
<td>[207]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High dose Ag alone&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Normal</td>
<td>NT</td>
<td>Enhanced</td>
</tr>
<tr>
<td>[125, 145]</td>
<td>([I, 124, 133])</td>
<td>[99]</td>
<td></td>
<td>[179]</td>
<td></td>
</tr>
<tr>
<td>Low dose Ag alone&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NT</td>
<td>Impaired</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>([I, 133])</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The Ab response is measured either as direct or indirect PFCs or as Ab titers in sera.
2 Mice were immunized with soluble Ag in adjuvant.
3 Mice were immunized with the particulate Ag in PBS.
4 NT, not tested.

**Table 2.** Feedback enhancement of Ab responses induced by ICs.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>C3 depleted</th>
<th>CR1/2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeRγ&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeγRIII&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>FeγRIIB&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM/Ag&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Impaired</td>
<td>Impaired</td>
<td>NT&lt;sup&gt;4&lt;/sup&gt;</td>
<td>NT</td>
<td>Normal</td>
</tr>
<tr>
<td>[89]</td>
<td>([I])</td>
<td></td>
<td></td>
<td>([III])</td>
<td></td>
</tr>
<tr>
<td>IgG1/Ag&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NT</td>
<td>NT</td>
<td>Impaired</td>
<td>Normal</td>
<td>Enhanced</td>
</tr>
<tr>
<td>([II])</td>
<td>([II])</td>
<td></td>
<td>([II, III])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG2a/Ag&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NT</td>
<td>Normal</td>
<td>Impaired</td>
<td>Normal</td>
<td>Enhanced</td>
</tr>
<tr>
<td>([I])</td>
<td>([II])</td>
<td></td>
<td>([II, III])</td>
<td></td>
<td></td>
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<tr>
<td>IgG2b/Ag&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Normal</td>
<td>NT</td>
<td>Impaired</td>
<td>Normal</td>
<td>Enhanced</td>
</tr>
<tr>
<td>[203]</td>
<td>([II])</td>
<td></td>
<td>([II])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG3/Ag&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Normal</td>
<td>NT</td>
<td>Normal</td>
<td>NT</td>
<td>Normal</td>
</tr>
<tr>
<td>([IV])</td>
<td>([IV])</td>
<td></td>
<td>([IV])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgE/Ag&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NT</td>
<td>Normal</td>
<td>Normal</td>
<td>NT</td>
<td>Enhanced</td>
</tr>
<tr>
<td>([I])</td>
<td>([II])</td>
<td></td>
<td>([II, III])</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 The Ab response is measured either as direct or indirect PFCs or as Ab titers in sera.
2 IgM-mediated enhancement was studied using SRBC and polyclonal IgM anti-SRBC.
3 IgG- and IgE-mediated enhancement was studied using soluble Ag in complex with a mAb.
4 NT, not tested.
Concluding remarks

From the results presented in the present investigation, the following conclusions can be made:

- IgM mediates enhancement of Ab responses via CR1/2. IgE- and IgG-mediated enhancement does not involve CR1/2, suggesting that these ICs circumvent the requirement for CR1/2 in the induction of Ab responses.

- IgG-mediated enhancement of Ab responses is most likely mediated via FcγRI although an overlapping function by FcγRIII cannot be excluded.

- FcγRIIB negatively regulates Ab responses to IgG/Ag complexes but not to IgE or IgM complexes. It probably acts by limiting the initiation of the B cell response rather than to completely suppress it.

- IgG3 can mediate enhancement of Ab responses to soluble Ags. This effect does not involve binding to FcγRI or FcγRIII. FcγRIIB does not inhibit the ability of IgG3 to enhance.

The physiological role of IgM-mediated enhancement is probably to act in the primary response, where IgM molecules mediate efficient priming of B cells via CR1/2. This ultimately leads to Ab switching and memory, when proper Th cell interaction has occurred. In the secondary response, when IgG is produced and present in the circulation, CR1/2 is most likely of less importance for the Ab response. Instead, specific IgG binds the Ag and forms ICs that interact with FcγRI which mediate phagocytosis leading to more efficient and rapid elimination of the Ag. Specific Abs function as "natural adjuvants" with the ability to direct the target Ag to the "right" effector cells. Both specific IgM and IgG may be used in the future to target Ag to CR1/2 or FcγRI, as efficient strategies for vaccination without involving conventional adjuvants.

IgG3 is formed early in immune responses. The ability of IgG3 to enhance Ab responses, without initial negative regulation by FcγRIIB, could be important for understanding how early IgG3 Abs can induce strong immune responses that may be of importance in the early defense against bacteria. The existence of IgG3 autoantibodies in disease models in mice (such as lupus and glomerulonephritis) [76, 175-177], implies that these Abs may further promote the production of pathogenic autoantibodies without involvement of FcγRIIB inhibition.
The fact that FcγRIIB−/− mice respond to very low doses of IgG/Ags could be an important mechanism in the development of autoimmune disease. Low doses of autoreactive Abs could up-regulate the production of more autoreactive Abs, provided that FcγRIIB had diminished inhibitory function owing to dysfunctional expression or mutations. In mice, polymorphisms both in the FcγRIIB encoding regions [168] and in the regulatory regions are found [98]. These are associated with susceptibility for spontaneous autoimmune diseases such as lupus and autoimmune diabetes [98, 168]. In humans, polymorphisms in FcγRIIa and FcγRIIIa have been shown to be associated with increased risk for systemic lupus erythematosus (reviewed in [193]).

An interesting idea would be to use FcγRIIB as a target for the treatment of autoimmunity. Targeting autoreactive B cells and causing crosslinking of BCR and FcγRIIB would efficiently eliminate these cells from the circulation. Measles virus uses a similar approach where the nucleocapsid protein of the virus can bind FcγRIIB and thereby probably reduces the production of Abs and escapes the immune system. [155]. In immunotherapy against tumors, the use of Abs that are unable to bind to FcγRIIB, but not to other FcγRs, could be more effective reagents. This approach has already been reported to function in a mouse tumor-model [34].
Acknowledgements

This work was carried out in the Department of Genetics and Pathology at the Rudbeck Laboratory, Uppsala University, Sweden. I wish to acknowledge the following people who, in different ways, have contributed to the completion of this thesis:

**Birgitta Heyman**, my supervisor, for excellent guidance, support and for always taking your time when I need to talk.

**Imma Brogren**, my "lab mother" for excellent technical assistance and for being such a nice person.

Former and present members of the lab: **Sara**, for introducing me to "Heyman-gruppen" and taking care of me when I was new in the lab; **Mickey**, for being a good travel companion and all the discussions about interesting ideas; **Teresita**, for clever questions and hypotheses; **Andy**, for being a calm and nice person with many bright ideas; **Sandra**, for being a nice person and all the interesting discussions; **Susanne**, for your impressive way of organizing things; **Pernilla**, for always being helpful and nice; **Sanna**, for those little "funny" notes you made in my notebook; **Steven**, for laughs and much more and **Maria**, for your enthusiasm and your positive attitude.

Finally, to all of you in the lab: for contributing to the good atmosphere, friendship and for putting up with my sense of humor by laughing at my "bad" jokes.

All the **Co-authors**, for fruitful collaborations.

All the people at the Department of Genetics and Pathology, for creating a positive and productive atmosphere. Especially, I want to thank the persons in the "genius-corner" in the "old" pathology building for interesting scientific as well as social discussions, the people at IT support, "polarn" **William** and the "newcomer" **Viktor** for sharing the same interest as myself for computers and all the computer-talks we have had during the years and the **secretaries** for helping me with administrative things.

Colleagues in the "Swedish family of immunologists" that I have met during congresses and courses especially people I met in Geilo 1996, New Delhi 1998 and San Francisco 1999, for laughter, friendship, interesting discussions and reunion-parties.

**Alan McWhirter**, for linguistic revision of the thesis.

My parents, **Aaby** and **Mona** and my brother, **Magnus** for always supporting me.
My wonderful wife, Sara and my lovely daughter, Johanna for all your love and support.

Financial support was provided by: The Ellen, Walter, and Lennart Hesselman's Foundation; Hans von Kantzow's Foundation; King Gustaf V:s 80 Year Foundation; The Swedish Medical Research Council; The Swedish Foundation for Health Care Science and Allergy Research; Agnes and Mac Rudberg's Foundation; Anna Cederbergs Foundation; Knut and Alice Wallenbergs Foundation.
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