Modulation of B cell access to antigen by passively administered antibodies

an explanation for antibody feedback regulation?

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Abstract

Antibody responses can be up- or down-regulated by passive administration of specific antibody together with antigen. Depending on the structure of the antigen and the antibody isotype, responses can be completely suppressed or enhanced up to a 1000-fold of what is seen in animals immunized with antigen alone.

IgG suppresses primary antibody responses against erythrocytes. Suppression works well in mice lacking Fc-receptors for IgG, C1q, C3, or complement receptor 1 and 2 (CR1/2). Here, we demonstrate that IgG anti-NP given to mice together with NP-conjugated sheep erythrocytes, suppresses the generation of NP-specific extra-foollicular antibody-secreting cells, NP-specific germinal center B cells, induction of memory and long-lived plasma cells. IgG increases antigen clearance but this does not explain the suppressed antibody response. It is demonstrated that IgG-mediated suppression of IgG responses is epitope specific, suggesting that epitope masking is the dominant explanation for IgG-mediated suppression of antibody responses.

Both IgE and IgG3 can enhance antibody responses against soluble antigens. IgE-antigen complexes bind to recirculating B cells expressing CD23, an Fc-receptor for IgE. Thirty minutes after intravenous administration, IgE-antigen is found in splenic follicles. Subsequently, germinal center responses, antigen-specific T cell proliferation, and antibody responses are enhanced. We show that also antigen conjugated to anti-CD23 can bind to CD23+ B cells and be transported to splenic follicles. CD11c+ spleen cells, rather than CD23+ B cells, present IgE-antigen complexes to T cells. Here, it is demonstrated that CD8α− conventional dendritic cells is the CD11c+ cell population presenting IgE-antigen to T cells.

IgG3-mediated enhancement is dependent on CR1/2. We find that IgG3-antigen complexes, administered intravenously to mice, bind to marginal zone B cells via CR1/2. These cells then transport IgG3-antigen into splenic follicles and deposit antigen onto follicular dendritic cells. Mice treated with FTY720, a drug which dislocates marginal zone B cells from the marginal zone, impairs this transport. Studies in bone marrow chimeric mice show that CR1/2 on both B cells and follicular dendritic cells are crucial for IgG3-mediated enhancement.

In summary, these observations suggest that antibodies can feedback regulate antibody responses by modulating the access of antigen to the immune system.

Keywords: IgG, IgG3, IgE, suppression, enhancement, epitope masking, antigen transport, antigen presentation, follicular B cells, marginal zone B cells

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No one is telling the truth.
No one knows the truth.
But try your best to get closer.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. H. Xu*, J. Bergström*, and B. Heyman. IgG-mediated suppression of IgG responses to erythrocytes is epitope specific. (manuscript) * equal contribution


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Abbreviations

Ab  antibody
Ag  antigen
ADAM10  a disintegrin and metalloproteinase 10
APC  antigen presenting cell
BCR  B cell receptor
Bcl-6  B cell lymphoma 6
Blimp-1  B lymphocyte-induced maturation protein-1
BrdU  Bromodeoxyuridine
BSA  Bovine Serum Albumin
C  complement
CCR  C-C chemokine receptor
CD  cluster of differentiation
CR  complement receptor
CXCR  C-X-C chemokine receptor
CXCL  C-X-C chemokine ligand
DC  dendritic cell
DTR  diphtheria toxin receptor
DZ  germinal center dark zone
Fc  fragment crystallizable
FcγR  Fc gamma receptor
FcεR  Fc epsilon receptor
FDC  follicular dendritic cell
FOB  follicular B cell
GC  germinal center
Ig  immunoglobulin
IC  immune complex
IL  interleukin
KO  knock out
KLH  Keyhole limpet hemocyanin
LZ  germinal center light zone
MHC  major histocompatibility complex
MOMA  metallophilic macrophage
MZ  marginal zone
MZB  marginal zone B cell
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>pMHC</td>
<td>peptide-loaded MHC molecule</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PALS</td>
<td>periarteriolar lymphoid sheath</td>
</tr>
<tr>
<td>RhD</td>
<td>rhesus D antigen</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine 1-phosphate</td>
</tr>
<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
</tr>
<tr>
<td>SIRPα</td>
<td>signal regulatory protein α</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Our immune system

During our lifetime, we are constantly surrounded by bacteria, viruses and parasites. To maintain health, our body has evolved an extraordinary system to fight against these pathogens: the immune system. When a pathogen is encountered, the first line of defense is the innate immune system. This consists of physical and chemical barriers, such as skin and mucus, and antibacterial proteins, such as complement (C) and serine proteases. Some leukocytes, including phagocytes (monocytes, macrophages), dendritic cells (DC), and natural killer cells, are also involved in innate immunity and recognize common structures on pathogens, so-called pathogen-associated molecular patterns (PAMPs). Many pathogens are eliminated during innate responses, and those that escape will be further handled by the adaptive immune system. The adaptive immune response reacts with high specificity against various pathogens and can “remember” these invaders. When it is exposed to the same pathogen again, it can respond with increased magnitude and prevent us from repeated infection. B cells and T cells are the major cell types that enable immunological memory responses.

Importantly, rather than operating separately, innate immunity and adaptive immunity are closely connected. For instance, the cytokines secreted by innate cells can stimulate T and B cells to differentiate into different effector cells, and antigen (Ag) taken up by DCs will later be presented on their major histocompatibility complex class II (MHC II) molecules to activate specific T cells. This sophisticated cooperation enables the immune system to keep us healthy.

An important feature of the immune system is the ability to regulate itself. Self- reactive B cells and T cells are silenced by negative selection through apoptosis or anergy (1), and specific antibody (Ab) binding to an Ag can feedback regulate the response to this Ag (2). This thesis focuses on the regulatory features of the immune system. Specifically, we have investigated how different Ab isotypes passively administered together with their specific Ags can regulate the response against these Ags. This is called Ab feedback regulation, and to study this phenomenon, Abs were typically injected intravenously into mice together with Ag; no adjuvants were used. Because the spleen is the major secondary lymphoid organ in which responses to blood-
borne Ags are initiated, this thesis mainly focuses on the immune responses in the spleen.

Structure of the mouse spleen

The mouse spleen can be divided into two parts, the red pulp and the white pulp (Fig. 1A), each of which has a different function. Its unique structure and position makes the spleen the primary site for the induction of immune responses to blood-borne Ags (3).

**Figure 1. Structure of the mouse spleen.** A. The white pulp is separated from and surrounded by red pulp. B. Structure of the red pulp with blood flowing in small arteries ending in cord structures, which are then collected by small veins. C. Structure of the white pulp where small arteries end in the sinus of the marginal zone. B cells and T cells are organized in different compartments.

Red pulp

The red pulp functions like a filter for the blood (4). Large arteries branch into smaller arteries and end in cord structures in the red pulp. These cords
are constructed by fibroblasts, and many red pulp macrophages reside here. The blood flow slows down here, where it is filtered and then collected into the efferent veins (Fig. 1B).

Red pulp macrophages can be identified with the marker F4/80, and a major function of these macrophages is to remove old erythrocytes through phagocytosis. The phagocytosed erythrocytes are destroyed in the lysosomes, and the iron is either released or stored for recycling (5). The iron recycling function is important for the body; it, as iron is a valuable mineral. In addition, this process can limit the growth of bacteria (6). Foreign erythrocytes are also removed by red pulp macrophages. As erythrocytes do not express MHC molecules, this is achieved via recognition of the CD47 molecule, which is an integrin-associated glycoprotein expressed on erythrocytes (7, 8). It can bind to receptor signal regulatory protein α (SIRPα) on red pulp macrophages and deliver inhibitory signals to the macrophages, preventing phagocytosis of the erythrocytes. Erythrocytes lacking CD47 are rapidly cleared (7). When mice are immunized with sheep erythrocytes (SRBCs) intravenously, the SRBCs are removed from the circulation within 10 mins (9), possibly by the red pulp macrophages. However, when SRBCs are complexed with specific IgG, clearance is even more rapid (8 and paper I).

White pulp
The white pulp is the area in which immune responses occur. Central arteries are sheathed by the T and B cell zones. The T cell zone is closest to the central arteries and is also known as the periarteriolar lymphoid sheath (PALS) (Fig. 1C). A small area of the T cell zone, called the marginal zone (MZ) bridging channel, extends into the red pulp, and a small population of DCs resides there (Fig. 2). DCs are regarded as the most professional Ag-presenting cells (APC), and this cell population is further discussed in paper III. The zone is also called the B cell follicle, or just follicle, and is filled with densely packed B cells. The border between the follicles and MZ sinus is defined by metallophilic macrophages (MOMA) (Fig. 2). Small branches of arteries, sometimes carrying blood-borne pathogens, end in an open sinus area, the MZ sinus (3). Different leukocytes, such as MZ macrophages, MZB cells and DCs, are localized in the MZ.

Two major B cell subsets, follicular B cells (FOB) and MZ B cells (MZB) can be found in the white pulp of the spleen. FOB cells circulate in the body through blood vessels and home to secondary lymphoid organs via chemokine attraction (10). MZB cells do not leave the spleen and are mainly resident in the MZ but can shuttle between the MZ and the follicles. This shuttling is also promoted by chemokines and chemokine receptors. FOB cells enter follicles in response to C-X-C chemokine ligand 13 (CXCL13), which
binds to C-X-C chemokine receptor 5 (CXCR5) on FOB cells. MZB cells use different chemokine receptors to enter or exit follicles. Migration into the follicle requires the chemokine receptor CXCR5, and return to the MZ requires the sphingosine 1-phosphate receptors S1P1 and S1P3 (11). When FTY720, an S1P antagonist, is administered to mice, the exit of MZB cells from the follicles can be inhibited. This inhibition results in a transient lack of MZB cells in the MZ (10). Both FOB cells and MZB cells have access to the splenic follicles, but the FOB cells recirculate through the body and capture Ag in the periphery, while the MZB cells capture Ag in the MZ. Therefore, together these two B cell types are ideal transporters of Ag "cargo" from the peripheral blood into splenic follicles.

Figure 2. | Confocal image of a section of a mouse spleen. The MZ metallophilic macrophages (green, stained for MOMA) line up along the inner edge of the MZ bordering on the B cell zone; B cells (blue, stained for IgD) and T cells (not stained) are separated into different compartments.

Ag transport to B cell follicles

Efficient humoral responses require that Ag is transported into secondary lymphoid organs, where it can interact with immune cells and initiate an immune response. How Ag is transported into follicles and encounters B cells has long remained elusive. Recent experimental studies using advanced techniques such as intravital microscopy have shed some light on this question.
Lymph-borne Ags and transport within lymph nodes

Small lymph-borne Ags (radius of approximately 4–5 nm or less than 70 kDa) can directly diffuse into lymphoid follicles via the gaps in the subcapsular sinus boundary (12) or can be transported to follicles through a conduit system (13). Particulate Ags, such as bacteria, can be enriched in the subcapsular sinus by subcapsular sinus macrophages, and FOB cells with high-affinity B cell receptors (BCRs) acquire the Ag directly from the macrophages and transport it into follicles (14). Subcapsular sinus macrophages can also capture C-opsonized IgG-Ag complexes, and FOB cells surveying the macrophage surface can capture the Ag via complement receptors (CRs) and then deposit the Ag on FDC (15). In addition, DCs can endocytose Ag via FcγRIIB, store Ag in non-degradative intracellular vesicles and then recycle it to the cell surface for interaction with Ag-specific FOB cells (16, 17).

Blood-borne Ags and transport within the spleen

Blood-borne bacteria are transported to the MZ by DCs and granulocytes, and the bacteria can then initiate T-independent responses via interaction with MZB cells (18). For example, an Fc chimeric protein containing the cysteine-rich domain of the murine mannose receptor and therefore capable of binding to macrophages and a subset of DCs in the MZ, was transported into B cell follicles when it was used as Ag (19-21). In addition to myeloid cells, both MZB cells and FOB cells are able to transfer blood-borne Ags into splenic follicles (Fig. 3). As mentioned before, MZB cells can shuttle between the MZ and the B cell follicles. The shuttling speed is rather quick, with 20% of cells being exchanged between the two compartments every hour (11, 22). MZB cells express high levels of CR1/2 and can therefore bind Ags, which are opsonized by C3 fragments. Ags in complex with IgM, which is an efficient activator of the classical C pathway, bind to CR1/2 on MZB cells and are transferred to follicles through the shuttling process (11, 23, 24). IgG3 can also activate C, and whether IgG3-complexed Ag can bind to MZB cells in a manner similar to IgM-complexed Ag is investigated in paper IV. FOB cells, which circulate through the body, can pick up virus-like particles from the lung via cognate BCRs and, via the blood, deliver the Ag to splenic follicles (25). FOB cells can also capture IgE immune complexes (IgE-IC) via the low-affinity IgE receptor CD23 and deposit the IgE-IC in follicles (26). IgE-mediated Ag transport is very rapid, and high amounts of Ag can be found in splenic follicles within 30 minutes of intravenous immunization. In addition, IgE-IC can also enhance ger-
minal center (GC) responses and T cell proliferation (26). In paper II, we evaluate whether this pathway can be utilized as an efficient vaccination method.

Figure 3. | B cell-mediated Ag transport to splenic follicles. A. MZB cells express CR1/2 and capture C-opsonized Ag, which is subsequently transferred to the follicle through MZB cell shuttling between the follicle and the MZ. B. FOB cells can capture Ag from the lung via their low-affinity BCRs and, via the blood stream, reach the follicles and deposit their Ag there. C. Circulating FOB cells express the low-affinity receptor for IgE, CD23, and can capture IgE-ICs in the blood and transfer them to splenic follicles.
B cell response to T-dependent Ags

After being activated by Ag binding to the BCR and receiving appropriate help, the specific B cell differentiates into different types of effector cells (Fig. 4). Here, the various steps in the B cell responses to T-dependent Ags, which are the main type of Ag used in the experimental set-ups, is discussed.

Pre-GC response

Once Ag-specific B cells are activated through the BCR-Ag interaction, these cells upregulate the level of chemokine receptor type 7 (CCR7) on their surface, which induces them to migrate towards the border between the T and B cell zones (27). In parallel, Ag-specific T cells are primed by APCs, mainly conventional DCs. Activated T cells upregulate C-X-C chemokine receptor type 5 (CXCR5), which guides them toward the T-B border (28). Here, Ag-specific B cells, which have endocytosed Ag, present Ag peptides on MHC II (pMHC) for recognition by Ag-specific T cells. Once pMHC on the B cell is engaged by T cells via the receptor (TCR), this cognate interaction together with other signals (such as CD40-CD40L) deliver survival signals to B cells, promoting B cell proliferation and differentiation.

After cognate interaction with T cells, B cells can differentiate through three pathways:

i) Upregulate the master transcription factor for plasma cells, Blimp-1 (B lymphocyte-induced maturation protein-1), and become short-lived plasmablasts/plasma cells (29-31). These cells exit B cell follicles, migrate to extra-follicular foci and secrete early Abs (30). These plasmablasts/plasma cells are mainly un-switched cells secreting IgM, but can also be switched and produce other Ab classes (32). As very little somatic mutation occurs in these plasmablasts/plasma cells, the Abs secreted in extra-follicular foci are usually of low affinity (32).

ii) Become GC-independent memory B cells and migrate back to B cell follicles. The majority of these early generated memory cells are IgM+ memory cells (33).

iii) Upregulate the master transcription factor for GC B cells, Bcl-6 (B cell lymphoma 6), and become GC B cells (34).

It is not yet clear which factor promotes the differentiation of B cells into memory cells versus GC B cells. CD40-CD40L interactions possibly play a role, as an agonistic anti-CD40 Ab enhances the size of the GC-independent memory cell population and inhibits GC formation (33).
GC response

GCs can be separated into a dark zone (DZ) and a light zone (LZ). When B cells are committed to become GC B cells, they first move to the DZ and proliferate extensively (35). During proliferation, GC B cells mutate their immunoglobulin variable regions at a very high mutation rate in a process called somatic hypermutation (36, 37). Subsequently, the B cells migrate toward the LZ of the GC, where the follicular dendritic cell (FDC) network is located. FDC are stromal cells that were recently shown to originate from ubiquitous perivascular precursors (38). These cells are known to express both Fc-gamma receptors and CR and can store immune complexes in a native state for extended periods of time (39, 40). It is assumed that the B cells with mutated BCRs compete with each other for Ags displayed on the surface of the FDC and that only B cells that have a high affinity are able to capture and endocytose Ag. According to recent data, this is not the only crucial step in the survival of GC B cells; they must also compete for a limited amount of help from T follicular helper cells (Tfh) by presenting pMHC to them (35). Logically, the two processes are interconnected, and only B cells that compete successfully for Ag on FDC are able to present pMHC to Tfh (41, 42). Full understanding of these processes requires further studies.

With the help of Tfh, GC B cells can also switch isotypes from IgM to one of the other Ig classes in a process called class-switch recombination (43, 44). Both class-switch recombination and somatic hypermutation are driven by the activation-induced cytidine deaminase (AID), and mice lacking this enzyme do not produce switched Ab classes, and their Ab response does not go through affinity maturation (45).

GC B cells that survive a first round of selection and competition as described above can differentiate via three pathways after interaction with Tfh cells:

i) Move back to the DZ and start another round of proliferation and selection (35).

ii) Upregulate Blimp-1 and become GC-dependent plasma cells (46, 47). These cells migrate to specialized niches in the bone marrow, where they remain and secrete Abs for long periods of time (48). As these plasma cells have mutated variable regions and have been selected through the GC reaction, they usually secrete Abs of high affinity (49).

iii) Become GC-dependent memory B cells (50, 51).

It is yet unclear how the decision to differentiate into either memory cells or plasma cells is regulated. Initially, affinity to the Ag was suggested to determine the fate of GC B cells, with high affinity, leading to the generation of GC-derived plasma cells, and low affinity inducing the generation of GC-
derived memory cells (52, 53). However, it was later demonstrated that high affinity promotes the proliferation of all Ag-specific B cells and not only differentiation into plasma cells (54). It has also been suggested that the decision to become memory or plasma cells could be a stochastic process (55). A recent study using bromodeoxyuridine (BrdU) to label memory cells and plasma cells at different time points proposed that memory cells are generated during the entire GC response period, while plasma cells are mainly generated during the later period of the GC response (56). Further studies are needed to confirm this hypothesis.
Figure 4. **B cell responses to T-dependent Ag.**

**A.** Ag-activated B cells upregulate CCR7 and migrate to the T-B border.

**B.** Ag-activated B cells present Ag peptides on MHC II and obtain help from Ag-primed T cells. These B cells then differentiate into GC-independent memory B cells or short-lived plasmablasts.

**C.** Alternatively, these B cells can become GC B cells and initiate a GC response.

**D.** The GC is separated into two zones: the light zone and the dark zone. GC B cells migrate between these two zones and compete for Ag localized on FDC with their refined BCRs.

**E.** GC B cells with high-affinity BCRs can obtain Ag from FDC, endocytose it and present peptides on their MHC II molecules and compete for help from limited numbers of Tfh cells. Only GC B cells obtaining sufficient signals from Tfh cells will survive and may re-enter the dark zone for another round of selection.

**F.** Surviving GC B cells can also exit the GC and differentiate into GC-dependent memory B cells or GC-dependent plasma cells.
Ab feedback regulation

Abs can feedback regulate the immune response when administered together with their specific Ag. Depending on the Ab isotype and the type of Ag, the effect can be either positive, causing a several 100-fold greater Ab response as compared to Ag alone, or negative, suppressing >99% of an Ab response (2, 57, 58). These effects take place in the absence of adjuvants, as Ab and Ag are administered in physiological salt solutions. An overview of the effects of different regulatory Abs is presented in Table 1.

<table>
<thead>
<tr>
<th>Passive Ab</th>
<th>Ag</th>
<th>Effect on Ab response</th>
<th>Effect on T cell response</th>
<th>Suggested mechanisms</th>
</tr>
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<tbody>
<tr>
<td>IgM</td>
<td>Particulate Ag, large soluble Ag</td>
<td>+++</td>
<td>No effect</td>
<td>Enhanced transport of IgM-C-Ag by CR1/2+ MZB cells for capture by FDC via CR1/2</td>
</tr>
<tr>
<td>IgG1, 2a, 2b</td>
<td>Soluble Ag</td>
<td>+++</td>
<td>+++</td>
<td>Enhanced Ag presentation via uptake of Ag by FcγRs</td>
</tr>
<tr>
<td>IgG3</td>
<td>Soluble Ag</td>
<td>+++</td>
<td>No effect</td>
<td>Enhanced transport of IgG3-C-Ag by CR1/2+ MZB cells for capture by FDC via CR1/2</td>
</tr>
<tr>
<td>IgG</td>
<td>Particulate Ag</td>
<td>---</td>
<td>-</td>
<td>Ag clearance and/or epitope masking</td>
</tr>
<tr>
<td>IgE</td>
<td>Soluble Ag</td>
<td>+++</td>
<td>+++</td>
<td>Enhanced IgE-Ag transport to follicles by CD23+ FOB cells</td>
</tr>
</tbody>
</table>

Table 1. Table 1 | Effects of Ab feedback regulation
(+++) enhancement, (---) suppression, (-) partial suppression
IgG-mediated suppression

It was discovered more than 100 years ago that passively administered Abs (or rather antiserum) can change the outcome of an Ab response to the Ag with which the antiserum reacted (59). Experiments using gradient centrifugation, which separates antiserum into different classes of Abs, demonstrated that IgG (then called 7S) is the isotype that suppresses responses to SRBCs, whereas IgM (19S) enhances the responses (60). IgG-mediated suppression is potent, and a small dose of SRBC-specific IgG administered together with SRBCs can suppress >99% of the early IgM response, as measured with a direct plaque-forming cell assay (60, 61). Secondary responses and induction of memory can also be suppressed, although usually to a lesser extent (62-65).

The mechanism behind the IgG-mediated suppression of the Ab response is still a mystery, although it has been used in the clinic since the 1960s to prevent hemolytic disease of the newborn (66, 67). RhD- mothers carrying RhD+ fetuses may develop anti-RhD Abs when fetal erythrocytes enter the maternal circulation via transplacental hemorrhage. IgG anti-RhD Abs are actively transferred to the fetus through the placenta and may cause hemolysis of fetal erythrocytes. Human studies in Liverpool by Ronald Finn and in New York by William Pollack and colleagues in the 1960s (immunizing RhD- male policemen and prisoners, respectively) demonstrated that passively injected anti-RhD Abs can efficiently prevent the development of an anti-RhD response to RhD+ red blood cells (RBC) (67, 68). After successful clinical trials, this method was quickly implemented to prevent Rh immunization in RhD- pregnant women (69). The anti-RhD Abs used for therapy are polyclonal Abs, mainly produced through the immunization of RhD- volunteers with RhD+ blood. Attempts to replace polyclonal anti-RhD with monoclonal anti-RhD Abs have so far been unsuccessful, though several monoclonal anti-RhD Abs have been tested clinically (70). A recent study revealed that a mixture of monoclonal Abs specific for different epitopes on the same erythrocyte could suppress the response as efficiently as polyclonal Abs (71). This finding may initiate clinical trials of combinations of several monoclonal IgG anti-RhD Abs and hopefully will result in more efficient suppression.

Several hypotheses have been proposed to explain IgG-mediated suppression:  

1) Central B cell inhibition via FcγRIIB. IgG in complex with particulate Ags such as erythrocytes may co-crosslink the BCR and the inhibitory FcγRIIB expressed on B cells. The cytoplasmic tail of FcγRIIB contains an inhibitory motif called the immuno-receptor tyrosine-based inhibition motif
Co-crosslinking of BCR and FcγRIIB is known to induce inhibitory signaling in the B cell (72-74) and could hypothetically result in unresponsiveness to the Ag, which in this case would be an erythrocyte.

ii) Elimination of IgG-Ag via FcγR-mediated phagocytosis or C-mediated lysis. IgG bound to erythrocytes may direct the Ag to phagocytes and increase clearance. Alternatively, IgG may recruit C factors and activate the classical pathway, resulting in Ag lysis. These two mechanisms would cause rapid removal of the Ag, and the specific B cells may not have time to recognize and bind to the Ag.

iii) Epitope masking. IgG binding to the Ag may simply hide Ag determinants, thus preventing B cells from recognizing the Ag.

Among these hypotheses, the first two would be dependent on a functioning IgG Fc region, whereas epitope masking would be Fc-independent. Several early studies suggested that the Fc portion was crucial for IgG-mediated suppression, as F(ab)\(2\) fragments were much less efficient suppressors than intact IgG (62, 75-77). However, other reports claimed that F(ab)\(2\) fragments were equally suppressive to intact IgG (78, 79). A possible explanation for the discrepancy may be that F(ab)\(2\) fragments are more rapidly removed from the circulation than intact IgG due to lack of protection from proteolysis by the neonatal FcR, FcRn (80).

To solve the question of Fc-dependence or not, Heyman and colleagues tested IgG-mediated suppression in various FcγR knock-out (KO) mice (81). IgG was equally efficient in suppressing Ab responses to SRBCs in FcγRIIB KO mice and in wild-type (WT) mice (81, 82), a result confirmed in more recent studies (83, 84). This strongly suggests that IgG-mediated suppression is not caused by central inhibition following the co-crosslinking of the BCR and FcγRIIB. Further studies showed that IgG efficiently suppressed this response in mice lacking FcγRI, III and IV (due to the lack of the common γ chain in FcR\(\gamma\) KO mice) (81, 83, 84) as well as in mice lacking CR1/2, C1q and C3 (84). These observations imply that FcR- and C-mediated Ag clearance are unlikely to be important mechanisms in IgG-mediated suppression.

Another interesting aspect of IgG-mediated suppression is whether the suppression is epitope specific or non-epitope specific. In other words, can IgG specific for one epitope of an Ag only suppress responses against this epitope or can it also suppress against other epitopes on the same Ag? Epitope-specific suppression would support the mechanism of epitope masking (iii above), whereas non-epitope-specific suppression has been thought to support the involvement of the Fc portion (i or ii above). Interestingly, experimental data demonstrating both epitope-specific suppression (85, 86) and non-epitope-specific suppression (9, 61, 76, 81, 87) have been presented. One possible explanation for these discrepancies is that epitope density plays
an important role. It is feasible that when the epitopes that IgG binds to are present at a low density, IgG will only cause epitope-specific suppression (Fig. 5A), whereas when the epitopes are present at a high density, IgG may also block the neighboring epitopes via steric hindrance (Fig. 5B) (9, 88). Experimental support for this idea was observed when TNP-specific IgG suppressed responses to SRBC determinants when administered with SRBC-TNP with a high TNP density, whereas poor suppression of responses to SRBC determinants was observed with low TNP density (89).

To address these uncertainties, we have here evaluated whether IgG-induced Ag clearance and/or epitope masking are involved in IgG-mediated suppression. Since the vast majority of earlier data on IgG-mediated suppression were generated using direct PFC as a read-out, (measuring only early IgM secretion by single cells), we have here analyzed the IgG-mediated suppression of long-term IgG responses, the induction of immunological memory and long-lived plasma cells. Finally, we were able to visualize NP-specific Ab-producing cells in the B cell follicles as well as in the extra-follicular area. These experiments are described in paper I.

**IgE-mediated enhancement**

The main function of IgE in the immune system is thought to be in responses against parasite infections (90). However, IgE is currently better known for its role in atopic diseases or allergies. In mice, as well as in humans, the following two receptors for IgE exist: the high-affinity receptor FcεRI and the low-affinity receptor FcεRII, or CD23. FcεRI is mainly expressed on mast cells and basophils. Allergic individuals who have been sensitized to allergen will have mast cells and basophils loaded with allergen-specific IgE bound to FcεRI. Upon re-encountering the same allergen, IgE cross linking leads to the degranulation of mast cells (91). Unlike all other FcRs, CD23
does not belong the super-Ig family, but belongs to the type-II C-type lectin family and is a membrane-bound trimer (92). This means it has the potential to bind carbohydrate structures. CD23 has two isoforms in humans. CD23a is constitutively expressed on B cells and FDC, while CD23b is expressed on epithelial cells and other inflammatory cells after induction by IL-4 (91). In mice, CD23a was thought to be the only isoform expressed on B cells and FDC, until recent studies revealed that CD23b can be found on intestinal epithelial cells and can transport IgE-IC from the apical surface to the basolateral face of the epithelial cells (93, 94). CD23 can be cleaved by ADAM10 (a disintegrin and metalloproteinase 10), released from the cell membrane and become a soluble molecule (95). Both the membrane-bound and soluble forms of CD23 were found to regulate IgE homeostasis (91).

Another important role of CD23 is to mediate the ability of IgE to feedback regulate Ab responses (96). When mice are given IgE anti-2,4,6-trinitrophenyl (TNP) together with ovalbumin (OVA) or bovine serum albumin (BSA) conjugated to TNP, the carrier-specific Ab response increases several 100-fold relative to when mice are given the Ags alone (26, 97-99). The primary IgG1, IgG2a, IgM, as well as IgE responses are elevated. In addition, GC responses, specific T cell responses and memory responses are all enhanced. These increased responses are absent when CD23 is removed from the system, as in CD23 KO mice (99-101) or when CD23 is blocked by anti-CD23 Abs (102, 103). Studies with IL-4 KO mice revealed that CD23a but not CD23b is crucial for the IgE-IC induced immune enhancement (104). To elucidate which CD23+ cell type is responsible for the enhancement, bone marrow chimeric mice were constructed. Mice with CD23−FDC and CD23+ bone marrow-derived cells responded well to IgE-ICs, while mice with CD23+ FDC and CD23− bone marrow-derived cells failed to respond. This indicates that CD23− B cells are playing the main role, while CD23+ FDC are dispensable (100). Further studies by Hjelm et al revealed that IgE anti-TNP in complex with biotin-OVA-TNP was detected on B cells in the blood 5 minutes after intravenous immunization. The complexes were also observed in splenic B cell follicles, where it was bound to FOB cells, after 30 minutes (26). The rapid Ag deposition in splenic follicles indicated the following possible explanation for the enhanced Ab and T cell responses induced by IgE-ICs: the IgE-ICs captured by CD23+ B cells and transported to the follicles may be endocytosed by these B cells and presented to T cells. This idea was supported by in vitro studies showing that B cells can indeed endocytose IgE-ICs via CD23 and subsequently present peptides to T cells (102, 103). However, whether B cells can present Ag to naïve T cells remains a controversial issue. Studies against (105-107) or in favor of (108-110) this idea are both reported. Moreover, the finding that peripheral CD23+ B cells transported IgE-ICs to the spleen suggested that the function of these cells could be to transport, rather than to present, IgE-ICs.
To test whether B cells indeed present IgE-ICs in vivo, a system in which CD11c<sup>+</sup> cells can be depleted was used (111). In CD11c-DTR mice, CD11c<sup>+</sup> cells can be conditionally depleted by the injection of diphtheria toxin. Data show that i) CD19-depleted splenocytes (mainly B cells are removed) from WT mice immunized with IgE-anti-TNP + OVA-TNP could induce T cell proliferation as well as the whole splenocyte population, while CD11c-depleted splenocytes (CD11c<sup>+</sup> cells are removed) from similarly treated WT mice failed to do so. ii) CD4<sup>+</sup> T cells from DO11.10 mice adoptively transferred to untreated CD11c-DTR mice showed increased proliferation after IgE + OVA-TNP immunization; when the mice were treated with diphtheria toxin to deplete the CD11c<sup>+</sup> cells, IgE + OVA-TNP did not induce an enhanced response. iii) Normal B cells from a mouse with a different MHC II than the recipient CD23 KO mice were able to restore IgE-induced T cell proliferation. Since Ag presentation requires the presenting B cells to have the same MHC as the responding T cells, these results suggested that CD11c<sup>+</sup> cells are the dominant APCs and that the main function of CD23<sup>+</sup> B cells is to transport IgE-ICs from the peripheral blood to splenic follicles. Since there are several DC subsets as well as other cell types that express CD11c, we found it interesting to more specifically identify which cell type is involved in the in vivo presentation of IgE-Ag complexes (paper III).

Targeting Ags to B cell-surface molecules other than CD23 was described to enhance Ag presentation (112) or Ab production (113). The mechanism suggested is that these surface molecules are capable of endocytosis. In support of this, targeting Ag to B220, a molecule that has no endocytic capacity, did not result in presentation to T cells (113). As described above, the binding of IgE-Ag complexes to CD23 can upregulate Ab responses. Instead of using IgE as a bridge between CD23 and Ag, the chemical conjugation of OVA to an anti-CD23 Ab and investigation of the response to OVA has been attempted. When such conjugates were administered subcutaneously, an increased immune response was observed (114). In paper II, we further investigated the effects of administering anti-CD23-OVA conjugates in vivo to mice.

**IgG-mediated enhancement**

IgG Abs have a dual effect in immunoregulation. When monoclonal IgG anti-TNP Abs are administered to mice together with SRBC-TNP, they suppress the SRBC response, and when they are administered with TNP-coupled proteins such as OVA, BSA, or KLH, they enhance the carrier responses (77, 89). Enhancement by murine IgG1, IgG2a, and possibly IgG2b is dependent on FcγRs rather than on C, as it is lost in mice lacking FcγRI, FcγRIII, and FcγRIV (115), whereas it remains normal in mice lacking...
CR1/2 (116). IgG2a increases the proliferation of Ag-specific CD4+ T cells when administered together with OVA-TNP (117, 118). This finding together with the important role of FcγRs, which must be expressed on CD11c+ cells (118), suggests that the mechanism underlying the IgG-mediated enhancement of immune responses is increased Ag-presentation to T cells.

In mice, IgG3 is the dominant subclass in the response to type 2 T-independent Ags but is less common in responses against T-dependent Ags (119, 120). IgG3-deficient mice are more susceptible to pneumococcal sepsis, indicating that IgG3 is important in the defense against bacterial infections (121). Unlike other IgG subclasses, IgG3 binds poorly to all FcγRs except FcγRI, to which it binds with low affinity (122, 123). Another unique feature of IgG3 is that it can self-associate through Fc-Fc interactions (124, 125). The ability to form large complexes probably facilitates the ability of IgG3 to activate C (126). When IgG3 is administered together with specific soluble Ags, the Ab response is highly elevated, sometimes up to 1000-fold (127, 128). Enhancement is equally efficient in FcγRIIB KO and FcγRγ KO mice and WT mice, whereas it is severely impaired in CR1/2 KO mice (127, 128). These experimental data demonstrated that the C system is crucial for IgG3-mediated enhancement. IgG3 was first thought to activate only the alternative pathway but was later shown to also activate the classical pathway (126).

The C system is crucial for humoral responses. This was first discovered when mice lacking C3 due to the injection of cobra venom factor had severely impaired Ab responses (129). Later, it was demonstrated that humans (130) and guinea pigs (131-133) lacking C2 or C4 also have poor Ab responses. Further studies using KO mice confirmed the importance of C3 and C4 in Ab responses (134). Interestingly, interruption of the alternative pathway by deleting factor B did not affect Ab responses (135). These observations indicate that classical, but not alternative, C pathway activation is important in regulating Ab responses. The classical pathway is mainly activated by IgM and IgG, and this activation leads to the cleavage of C3 into C3 fragments, which are the ligands for CR1/2. In mice, CR1 and CR2 are different splice variants encoded by the CR2 gene. Blocking CR1/2 with a CR1/2 specific Ab (136, 137) or removing the receptor via gene KO (138-140) severely impair the Ab responses.

Both the IgM- and IgG3-mediated enhancement of Ab responses are dependent on C and CR1/2 (127, 141-143). In paper IV, we sought to determine whether CR1/2 expressed on B cells and/or FDC is important for the ability of IgG3 to enhance Ab responses. Moreover, we wanted to elucidate the mechanisms responsible for the enhanced responses, e.g., whether IgG3-ICs are transported to splenic follicles by MZB cells.
Present investigation

The question I have addressed in this thesis is whether the mechanisms of Ab feedback regulation can be explained by Abs changing the accessibility of Ag to B cells. Passively administered Abs can either focus Ag towards B cells, which induces enhancement, or hide Ag from B cells via epitope masking, which causes suppression. Specifically, I have tried to answer following questions in each paper:

**Paper I.** Can IgG increase Ag clearance, and can this explain the suppressed Ab response?

Does IgG against certain epitope(s) on an Ag also suppress responses against other epitopes on the same Ag?

Can IgG suppress the generation of Ag-specific GC B cells, extra-follicular plasma cells, long-lived plasma cells and the induction of a memory response?

**Paper II.** Can anti-CD23-Ag conjugates direct Ag to CD23+ B cells in the blood, which then transfer the Ag to splenic follicles?

Can the primary and secondary Ab responses against the Ag be enhanced by anti-CD23-Ag conjugates?

**Paper III.** Which subsets of CD11c+ cells in the spleen are responsible for presenting IgE-immune complexes to T cells?

**Paper IV.** How are IgG3-ICs transported to splenic follicles?

Which cell type expressing CR1/2 is required for the IgG3-mediated enhancement of Ab responses?
Experimental setup - an overview

Mice
All experiments were conducted in the following strains of mice: BALB/c, C57BL/6, CD23 KO, CR1/2 KO, and DO11.10 (mice with CD4+ T cells expressing a transgenic TCR that recognizes the OVA peptide in I-A^d). All KO/transgenic mice were on a BALB/c background. Bone marrow chimeras between CR1/2 KO and WT mice were constructed by first sublethally irradiating recipient mice and then transferring the appropriate bone marrow intravenously.

Immunizations
Mice were immunized intravenously with either Ag-Ab conjugates or Ag complexed with specific Abs. When SRBCs or NP-SRBCs were used as Ag, Ab was administered 30 minutes prior to Ag to avoid agglutination.

Ag tracking, Ag localization, and measurement of Ab responses
To track the Ag and determine Ag localization, biotinylated Ag alone or in complex with Abs was administered to mice, and blood and spleen samples were harvested at different time points after immunization. Samples were prepared and stained with streptavidin-conjugated fluorophores, and the stained samples were analyzed with flow cytometry and confocal microscopy. Ag-specific Ab responses were detected with enzyme-linked immunosorbent assays (ELISA), and the number of Ag-specific plasma cells was measured with enzyme-linked immunospot (ELISPOT) assays or plaque-forming cell assays (PFC).

Detection of Ag-specific cells in the spleen
To visualize NP-specific cells, C57BL/6 mice were immunized with NP-SRBCs. Spleens were harvested 6 days after the immunization, stained with
NP-coupled phycoerythrin and then detected with confocal microscopy. When flow cytometry was used to analyze Ag-specific cells, both NP-coupled phycoerythrin and anti-\(\lambda\)1 were used.

T cell proliferation in vivo and in vitro

T cell proliferation was analyzed in vivo in mice into which DO11.10 T cells were transferred: the mice were immunized the day following the transfer. Three days after immunization, spleens were removed and OVA-specific T cells were visualized with the KJ1-26 monoclonal Ab, which recognizes the OVA-specific T cell receptor on DO11.10 T cells. T cell proliferation was evaluated in vitro by co-culturing different DC populations with CFSE-labeled DO11.10 T cells.
Results and discussion

Paper I

IgG increases Ag clearance, but this cannot explain the lack of an Ab response

It has been shown that IgG can decrease Ag localization in the MZ of the spleen (9). To confirm this, mice were immunized with 5x10⁷ NP-SRBCs together with 30 µg anti-NP IgG or with 5x10⁷ NP-SRBCs alone. In agreement with the previous study (9), IgG caused a decrease in Ag concentration in the MZ (Fig. 1I, paper I). The amount of Ag in the groups given 1x10⁷ NP-SRBCs was similar to that in the groups given IgG and the 5-fold higher Ag dose, 5x10⁷ NP-SRBCs. In spite of this, the responses to anti-SRBC IgG differed dramatically: whereas the mice immunized with IgG together with a high dose of NP-SRBCs had a completely suppressed response, the mice immunized with the low dose of NP-SRBCs responded almost as efficiently as the mice given the high dose alone (Fig. 1J, paper I). The decreased Ag load in our experiments is possibly caused by Fc-dependent phagocytosis in red pulp macrophages.

Earlier studies have shown that SRBCs are removed from the circulation of mice within 10 minutes and that IgG further increases the clearance rate (9). Studies in humans, in which monoclonal anti-RhD Abs were administered together with RhD⁺ RBC to RhD⁻ subjects, demonstrated that although some monoclonal anti-RhD Abs cause the rapid clearance of RBCs, the Ab responses were not suppressed. In a recent study of IgG-mediated suppression in mice, suppression was always induced, regardless of whether the monoclonal IgG Abs induced clearance or not (144). These data, together with our current findings, indicate that IgG-mediated Ag clearance is not the dominant mechanism behind suppression.

IgG suppresses only epitope-specific IgG responses

NP-SRBC was used as an Ag to test whether IgG-mediated suppression is epitope specific or non-epitope specific. Either anti-NP IgG or anti-SRBC IgG were used as suppressive Abs. Mice were bled every 2 weeks, and serum IgM and IgG responses against both SRBCs and NP epitopes were ana-
alyzed with ELISA. When looking at the IgM response, anti-NP IgG only suppressed responses against the NP epitopes (epitope-specific), whereas anti-SRBC IgG suppressed responses against both the NP and SRBC epitopes (non-epitope-specific). Earlier studies using PFC to measure the early IgM response were also ambiguous, reporting both epitope-specific (85, 86) and non-epitope-specific (61, 87, 89, 145) suppression. As mentioned before, a crucial factor to consider in these discrepancies is the epitope density (70 and Fig. 5). NP is a rather small molecule that, when coupled to SRBCs, may leave sufficient space for cognate B cells to interact with the SRBC surface epitopes (Fig. 5A). On the other hand, SRBC epitopes are surface proteins that are much larger and presumably more abundant on the erythrocyte surface than NP. Therefore, when SRBC epitopes are covered with passively administered IgG, there may be very little space for cognate B cells to reach the NP epitopes on the SRBC surface (Fig. 5B). Interestingly, when looking at the IgG response, the following striking epitope-specific suppression was observed: anti-NP IgG only suppressed responses against NP and left the SRBC responses untouched. Vice versa, anti-SRBC IgG suppressed only the SRBC responses and did not affect the NP responses.

Why does the suppression of IgG responses appear to be strictly epitope specific while the suppression of IgM responses in some situations is non-epitope specific? A possible explanation could be that B cells producing IgG generally are GC processed and have a higher affinity than B cells producing IgM early in the immune response. The high-affinity BCR enables the late (IgG-producing) B cells to compete more efficiently for epitopes that are sterically hindered by the passively administered suppressive IgG Abs (and not accessible to low affinity IgM-producing B cells). As an example, late high-affinity, NP-specific B cells producing anti-NP IgG may be able to bind NP epitopes on NP-SRBCs even in the presence of suppressive anti-SRBC IgG. Thus, the production of anti-NP IgG can occur, and epitope-specific suppression results. On the other hand, early low-affinity, NP-specific B cells producing IgM may not be able to bind NP epitopes on NP-SRBCs when the suppressive anti-SRBC IgG has bound and sterically hinders these B cells. This scenario would result in non-epitope-specific suppression of IgM responses because neither SRBC-specific nor NP-specific B cells can bind the Ag. In addition, after being deposited on FDC, IgG-Ag complexes are stored in non-degradable vesicles periodically recycled to the FDC surface (40). This recycling may change the conformation of the immune complexes and expose previously hidden epitopes.
IgG suppresses the generation of extra-follicular and Ag-specific GC B cells as well as long-lived plasma cells and the induction of immunological memory

Since it was first described more than 100 years ago (59), the mechanism behind IgG-mediated suppression has been extensively studied. Data regarding IgG-mediated suppression have mainly been generated from mice immunized with SRBCs together with anti-SRBC IgG, and the read-out has been PFC measured 5 days after immunization. This assay only detects the early IgM response. As mentioned before, Ag-activated B cells can also differentiate into early memory B cells or class-switched plasmablasts and can also enter GCs to become GC-processed memory B cells or plasma cells (36, 146-148).

To evaluate how IgG can affect the generation of Ag-specific GC versus non-GC B cells as well as Ab-secreting cells in extra-follicular foci of the spleen, C57BL/6 mice were immunized with anti-NP IgG together with NP-SRBCs. Spleens were harvested on day 6, and NP-binding cells were analyzed with confocal microscopy and flow cytometry. A small population of NP-specific cells were clearly identified in flow cytometry analysis of spleens (Fig. 3, paper I). These cells constituted 0.05% of the total population of B220+ cells and were present both in the GC and non-GC populations. Using confocal microscopy, the NP-specific cells were found to be mainly localized in extra-follicular foci. A few NP-specific cells could also be identified inside the GCs but were stained much less intensely than the extra-follicular NP-specific cells. To our knowledge, this is the first time that Ag-specific B cells have been visualized in vivo after the immunization of normal (not Ig-transgenic) mice without adjuvants.

Next, we sought to determine how IgG affected the immune response during longer time periods. Mice were immunized with SRBCs ± IgG, and SRBC-specific plasma cells in the spleen and bone marrow were analyzed in ELISPOT assays over the course of 10 weeks (Fig. 4, paper I). IgG completely suppressed the generation of SRBC-specific plasma cells at all times. It is harder to efficiently suppress the induction of immunological memory with IgG than it is to efficiently suppress a primary response (64, 149). Here, we primed mice with two different doses of SRBCs together with anti-SRBC IgG and boosted them with suboptimal amounts of SRBCs 10 weeks after priming (Fig. 5, paper I). IgG suppressed >97% of the memory response. Interestingly, a small memory response was still present even in the IgG-suppressed mice since they respond slightly more to the booster dose of SRBCs than do mice that received the same dose as a primary injection.
Taken together, these data show that IgG efficiently suppresses the generation of Ag-specific cells in GCs and extra-follicular foci, long-lived plasma cells and the induction of immunological memory. The small memory response in the suppressed groups is possibly due to poorly suppressed T cell priming (81). However, a small fraction of memory B cells may also be refractory to suppression by IgG.

Concluding remarks (paper I):
The epitope masking model for IgG-mediated suppression is the most likely explanation for the observations presented in paper I as well as for the majority of previously published data. This conclusion is primarily based on the following considerations.

i). The epitope specificity of suppression, clearly shown for the IgG-responses in paper I, is hard to reconcile with any of the other hypotheses for IgG-mediated suppression. It is feasible that steric hindrance by suppressive IgG in some situations explains why the non-epitope-specific suppression of early IgM-responses has also been observed.

ii). Suppression occurs efficiently in the absence of FcγRs and C.

iii). IgG is a very poor suppressor of T helper cell induction in the same mice in which the Ab response is completely suppressed.

However, some results seem difficult to explain with epitope masking. For example, if epitope masking is the primary mechanism explaining IgG-mediated suppression, why does IgM cause enhancement rather than suppression (60, 143, 150)? The simplest explanation would be that low-affinity IgM is less efficient at blocking epitopes and competing with cognate B cells than the high-affinity IgG that is used to suppress responses. IgM Abs administered with particulate Ags or large soluble Ags can active C, and therefore the IC can bind to CR1/2 on B cells and FDC (151). This increases the chance that the IgM-Ag-C complexes will encounter cognate B cells and lowers the threshold for B cell activation by co-crosslinking BCR and CR2. C-opsonized Ags will also be efficiently transported from the MZ to the B cell follicles by MZB cells expressing high levels of CR1/2 and will be deposited onto FDC that also express CR1/2 (11, 23). Thus, presumably the IgM-SRBC-C complexes are transported into follicles and deposited onto FDC while the epitope masking effect (owing to the low affinity of IgM) is very weak, explaining why IgM usually causes enhancement instead of suppression of the Ab response. When large amounts of IgM were administered (where the epitope-masking effect is dominant), suppression was induced (152, 153). Another study demonstrated that monoclonal anti-NP IgM could suppress the generation of NP-specific plasmablasts (154). In that study, NP-
CGG in alum was used as the Ag, and IgM was administered after Ag, making it difficult to compare their data with our data. IgM given after the Ag cannot increase Ag deposition on FDC and therefore, the effect of IgM in this situation will be epitope masking and the result will be suppression (154, 155).

IgG3 administered together with soluble Ag is known to cause the enhancement of Ab responses due to the C-mediated localization of Ag onto FDC (paper IV). It would be interesting to see whether administering IgG3 1 day after soluble Ag will suppress instead of enhance the Ab response. However, small soluble Ags are poor immunogens and usually do not induce a detectable Ab response when administered without adjuvants. However, a large Ag such as keyhole limpet hemocyanin (KLH) could be used to validate this hypothesis.

In addition to addressing the mechanism behind IgG-mediated suppression, paper I for the first time shows that IgG is able to suppress many more parameters of the Ab response than the primary IgM response that has been the focus of earlier studies. These data provide a more comprehensive understanding of IgG-mediated suppression.

Paper II

**Anti-CD23-OVA conjugates enhance Ag binding to B220^+ cells in blood and increase Ag localization in splenic follicles**

When injected intravenously to mice, anti-TNP IgE can direct OVA-TNP to B220^+ cells in the peripheral blood, and CD23^+ B cells can subsequently transport the IgE-IC into splenic follicles (26). Here, without using IgE, conjugates of OVA and monoclonal anti-CD23 Ab were used to test whether these conjugates could achieve the same function as IgE-Ag. As described in paper II, 5 min after immunization, the majority of B220^+ cells in the blood are loaded with anti-CD23-OVA conjugates. Thirty minutes after immunization, the anti-CD23-OVA conjugates were found in high concentration inside splenic follicles (Fig. 4, paper II). Flow cytometry analysis of the spleen confirmed that the majority of B220^+ cells in the spleen were binding to the conjugates (Fig. 3, paper II). In contrast, no OVA binding to B220^+ cells in the spleen or blood was observed in WT mice treated with OVA conjugated to an isotype control Ab (iso-OVA) or in CD23 KO mice treated with anti-CD23-OVA or iso-OVA. These data imply that Ag administered either in complex with IgE or chemically coupled to anti-CD23 is directed to and captured by CD23^+ B cells and then transported to B cell follicles. This is consistent with an earlier in vitro study showing that anti-CD23-OVA
conjugates can enhance both B cell proliferation and the presentation of these conjugates to T cells (114) and is also analogous to what was observed previously after the administration of IgE-ICs (26).

Ag deposition on FDC is a crucial step in affinity maturation of the Ab response (156, 157). In order to be loaded onto FDC, Ag first needs to obtain access to splenic follicles. Small Ags can be transported to splenic follicles through conduits (158, 159). Larger Ags do not have free access to splenic follicles, and either facilitating molecules or transporter cells are required. Several mechanisms have been reported for how B cells can mediate the transport of Ags into splenic follicles. Intranasally administered virus-like particles can be captured by recirculating B cells through their low-affinity B cell receptors and be transported to the spleen (25). MZB cells, which express high levels of CR1/2, can recognize C-opsonized Ag. Since these cells constantly shuttle between splenic follicles and the MZ, they can transfer opsonized Ag into follicles (11). Finally, recirculating B cells can capture IgE-ICs via CD23 and transport it to follicles (26). As shown in paper II, anti-CD23-OVA can also bind to CD23 on recirculating B cells and be transported to splenic follicles, thus confirming and extending previous findings.

**Anti-CD23-OVA conjugates enhance primary but not memory Ab responses**

The ability to induce several-hundred-fold higher Ab responses and T cell activation, demonstrated previously, suggests that IgE may be a potent adjuvant for vaccination. However, the risk of inducing anaphylaxis and allergic disease prevents it from being used. Moreover, the requirement of Ag-specific IgE to form the IgE-ICs makes it difficult to use in practice. By using anti-CD23-OVA conjugates, Ag can be conjugated to anti-CD23 regardless of Ab specificity, and IgE does not have to be used. Here, we demonstrate that anti-CD23-OVA can stimulate a 5-fold higher OVA-specific Ab response than iso-OVA conjugates and that no enhanced memory response was observed (Fig. 5, paper II). In a previous study, anti-CD23-OVA administered subcutaneously to mice induced detectable IgG1 and IgE primary responses and an enhanced memory response (114). A possible explanation for the lack of a memory response in our study could be the different methods of immunization, as Ag is more rapidly cleared from the blood than from the skin.

Concluding remarks are presented below for paper II and paper III together.
Paper III

**Anti-OVA IgE enhances OVA-specific immune responses and the localization of OVA in splenic B cell follicles**

In this paper, we used monoclonal anti-OVA IgE and OVA instead of the anti-TNP IgE and TNP-conjugated carrier proteins that were used in previous studies (26). OVA can more easily be conjugated to fluorophores without destroying important epitopes than can OVA-TNP and allows better visualization of the Ag in vivo. Monoclonal anti-OVA IgE enhanced both OVA-specific Ab and CD4+ T cell responses in WT mice but not in CD23 KO mice (Fig. 1, *paper III*). This was analogous to the results of previous studies using anti-TNP IgE and OVA-TNP. Fluorescently labeled OVA bound to FOB cells and was detected inside follicles in WT mice 0.5 h after administration together with anti-OVA IgE (Fig. 2, *paper III*). The amount of Ag increased and persisted until at least 4 h after immunization. However, in mice immunized with OVA alone, very little Ag was observed inside B cell follicles.

**CD8α− cDCs present IgE-complexed Ag to CD4+ T cells**

It has previously been shown that CD11c+ cells, but not B cells, are the APCs that present IgE-Ag to CD4+ T cells in vivo (111). The following are the three major subsets of CD11c+ cells in the mouse spleen: CD8α− cDCs, CD8α+ cDCs, and pDCs (160). To decipher which subset is the dominant cell type presenting IgE-complexed Ag to CD4+ T cells, we sorted these three subsets of CD11c+ cells by fluorescence-activated cell sorting splenocytes from mice immunized with IgE-OVA complexes or OVA alone. These cells were used as APCs in co-cultures with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OVA-specific CD4+ T cells. Cell cultures were incubated for 3 days before analysis of T cell proliferation with flow cytometry. We found that among these three subsets, only CD8α− cDCs from mice immunized with IgE-OVA complexes or OVA alone. These cells were used as APCs in co-cultures with 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled OVA-specific CD4+ T cells. Cell cultures were incubated for 3 days before analysis of T cell proliferation with flow cytometry. We found that among these three subsets, only CD8α− cDCs from mice immunized with IgE-OVA complexes efficiently induced Ag-specific CD4+ T cell proliferation. This was confirmed using another specific marker, DCIR2 (expressed on > 94% of all CD8α+ cDCs), to sort CD8α− cDCs. The other two subsets of DCs primed CD4+ T cells poorly, regardless of whether they were from mice immunized with IgE-OVA complexes or OVA alone. Importantly, the CD8α− cDCs did not express CD23, which suggests that they acquire Ag through other pathways.

**CD8α− cDCs migrate from the MZ bridging channels into the T cell zone after immunization**

Resting CD8α− cDCs reside in the MZ bridging channels, a connection in the MZ envelope penetrating from the white pulp into the rep pulp of the spleen (161). They migrate into the T cell zone after being stimulated by microbes
or microbial components such as LPS (162). We found that immunization with either IgE-OVA complexes or OVA alone induced the migration of CD8α- cDCs from the MZ bridging channels into the T cell zone. Migration was most prominent at 8 h after immunization (Fig. 5, paper III). Consistent with this, the ability of splenocytes from mice immunized with IgE-OVA complexes to present Ag to CD4+ T cells also peaked 8 h after immunization. Moreover, CD8α- cDCs from mice immunized with either IgE-OVA complexes or OVA alone upregulated the expression of CD86 and MHC II, which is a sign of activation. In contrast, CD8α- cDCs from mice immunized with IgE alone or with PBS exhibited CD86 and MHC II levels that were as low as those in unimmunized mice. However, it is still not clear how CD8α- cDCs are activated. It is possible that the OVA preparation was contaminated with certain components that bind to TLR1/2 or TLR4 on CD8α- cDCs, thus activating the cells and inducing them to migrate into the T cell zone. Here, they presumably encounter Ag-positive B cells and acquire Ag through unknown mechanisms.

**Concluding remarks (papers II + III)**

CD23+ B cells can rapidly transport IgE-ICs to splenic follicles and enhance both Ab and T cell responses tremendously. In paper II, we demonstrated that anti-CD23-OVA conjugates function similarly to IgE-ICs. They can also direct Ag to CD23+ B cells and be rapidly transported into splenic follicles.

Compared to the several-hundred-fold enhanced Ab responses that can be induced by IgE-ICs, anti-CD23-OVA stimulated a much lower enhancement (5-fold). One explanation could be the different endocytosis processes utilized by B cells after the Ag were captured. An in vitro study reported that anti-CD23 are endocytosed and degraded, while IgE are recycled to the cell surface after endocytosis (163).

The main role of recirculating B cells in IgE-mediated enhancement is to transport IgE-ICs to splenic follicles while CD11c+ cells present the Ag to T cells. The mechanism by which IgE-ICs are transferred from B cells to CD11c+ cells is still a mystery. One explanation may be that B cell-derived exosomes can function as a vehicle for transferring IgE-ICs to CD11c+ cells (164). Another possibility is that the increased local concentration of Ag close to DCs resulting from CD23+ B cell-mediated Ag transport would be sufficient to elevate Ag presentation by DCs to T cells. In addition, the binding between CD23 and IgE is calcium dependent (165, 166), and low calcium concentrations leads to the release of IgE-ICs from CD23+ B cells. Therefore, it would be interesting to test the local calcium concentration in the white pulp of the spleen.
Unlike other Fc receptors, CD23 is a member of the type II c-type lectin family, which has the potential to bind to carbohydrate structures (92). In fact, CD23 has been reported to bind to mannose structures and galactose (92, 166, 167). Presumably, Ags containing these structures could bind to CD23 and be transported to follicles without the involvement of IgE. We have expressed murine CD23 molecules on HEK 293 cell lines and tried to screen for bacterial strains that could possibly bind to CD23. Unfortunately, no ligands have yet been found.

CD11c+ cells were demonstrated to be the dominant APCs during the IgE-mediated enhancement of immune responses (111). In paper III, we rule out the involvement of other CD11c+-expressing cells and demonstrate that CD8α- cDCs are the principle APCs in IgE-mediated enhancement.

One unexpected observation was that although the Ab responses in mice immunized with OVA alone were extremely low, the CD8α- cDCs in these mice migrated to the T cell zone equally as well as those in mice immunized with IgE-ICs. CD8α- cDCs in the MZ bridging channels move into the T cell zone upon stimulation with LPS, Toxoplasma gondii or high doses of SRBCs (162, 168, 169). One possible explanation for our observation is that the migration of the CD8α- cDCs was induced by endotoxin in the OVA preparations. CD8α- cDCs from OVA-alone immunized mice did not increase T cell proliferation, indicating that these CD8α- cDCs did not present sufficient amounts of OVA peptide to T cells. CD8α- cDCs in mice immunized with IgE-ICs enhanced T cell proliferation, although CD8α- cDCs from both groups migrated to the T cell zone. Thus, only when sufficient amounts of Ag are transported and presented in follicles can CD8α- cDCs acquire Ag, endocytose it and stimulate T cell priming. This highlights the importance of the role of CD23+ B cells in transport: no Ag, no presentation to T cells - even if the CD8α- cDCs are present in the T cell zone.

Paper IV

MZB cells capture IgG3-ICs and transport them to splenic follicles

Anti-TNP IgG3 was administered intravenously together with OVA-TNP (IgG3-IC), and the binding of Ag to various B cells was analyzed. We observed that IgG3 does not facilitate the binding of Ag to circulating B cells (Fig. 1, paper IV), and no IgG3-IC was found on non-B lymphocytes (data not shown). In the spleen, IgG3-ICs were detected on MZB cells to a much higher degree than when the Ag was given without IgG3 (Fig. 2E, paper IV). FOB cells in the spleen do not bind to IgG3-ICs (Fig. 2F, paper IV).
CR1/2 KO mice, Ag alone localized on MZB cells in spleen, but IgG3 did not increase this binding (Fig. 2E, paper IV). Analysis with confocal microscopy revealed that IgG3-ICs, but not Ag alone, are present inside the follicles of WT mice, while neither IgG3-ICs nor Ag alone reached the follicles in CR1/2 KO mice (Fig. 3, paper IV).

As MZ B cells shuttle between the MZ and the B cell follicles (11), it appeared possible that MZB cells could capture IgG3-ICs in the MZ and transport them to the follicles. To directly test this, WT mice were treated with FTY720 to dislocate MZB cells from the MZ and were then immunized with IgG3-ICs or Ag alone. As expected, a severe reduction in the level of IgG3-ICs on the surface of MZB cells was detected in the FTY720-treated mice (Fig. 5, paper IV).

IgG3 enhances Ag localization on FDC and induces GC formation
Ags localized on FDC are important for the ensuing GC reaction. As large amounts of Ag are transported to splenic follicles when IgG3-ICs are administered to mice, we tested whether the Ag inside follicles co-localized with FDC. Ag was identified on FDC as early as 2 h post immunization, and low amounts of Ag still co-localized with FDC 8 h after immunization (Fig. 4, paper IV). These observations are in line with recent findings that FDC retain Ag on their surface, endocytose it into non-degrading endosomes and recycle Ag to the surface again, thus generating “Ag libraries” (40). Ten days after priming, spleens were harvested and GC formation was analyzed. Large GCs were observed in IgG3-IC treated mice, but very few GCs were observed in the Ag-only group (Fig. 6, paper IV).

CR1/2 expression on both MZ B cells and FDC is required for optimal Ab production after immunization with IgG3-ICs
CR1/2 are expressed on both B cells and FDC in mice. We generated bone marrow chimeric mice to evaluate how each of these cell types contributed to the Ab response after immunization with IgG3-ICs. As expected, IgG3 was able to enhance Ab responses in mice that expressed CR1/2 on both FDC and B cells. When CR1/2 was missing from either cell type, Ab production was reduced but was still higher than that in mice lacking CR1/2 on both B cells and FDC. Taken together, these observations suggest that CR1/2 on both B cells and FDC are required for optimal Ab production in response to IgG3-ICs.

Concluding remarks (paper IV)
The unique capacity to self-associate via Fc-Fc interactions probably enables IgG3 to activate the C system more easily than other IgG subclasses and makes it more like an IgM molecule. In paper IV, we found that IgG3-ICs
do not bind to FOB cells in the blood but bind to MZB cells provided that they express CR1/2. Presumably, IgG3 in complex with Ag can recruit other IgG3 molecules and activate the C system, which leads to the binding of IgG3-ICs to CR1/2 on MZB cells. Even in the absence of immunization, MZB cells constantly shuttle between the MZ and B cell follicles (11). Here, we demonstrate that IgG3-ICs, after binding to MZ B cells, are transported into splenic follicles and deposited onto FDC. Using bone marrow chimeric mice, we further showed that CR1/2 expression on both FDC and MZB cells is needed to achieve optimal enhancement, implying that transportation by MZB cells and deposition on FDC are equally important. Similar observations were made in IgM-mediated enhancement, with a lack of CR1/2 on either MZB cells or FDC reducing the level of enhancement (151). Interestingly, in both situations, it seems that a lack of CR1/2 on MZB cells but not on FDC mainly reduces the early Ab responses, while the enhancement is less affected at later time points. On the contrary, when CR1/2 is missing from FDC but remains on MZB cells, Ab enhancement is more intact at early time points but decreases at later time points. A possible explanation for this would be that, in situations with CR1/2− MZB cells and CR1/2+ FDC, the transportation of IgG3-ICs or IgM-ICs via MZB cells increase Ag concentrations quickly after immunization, and this enhances the early Ab response. However, when CR1/2 is missing from FDC, the ICs are removed from the body, and thus the later responses, decrease. In situations with CR1/2− MZB cells and CR1/2+ FDC, IgG3-ICs or IgM-ICs can only be slowly transported into follicles and therefore early enhancement is very low. Since FDC can continuously capture ICs and store them for extended periods of time, the enhancement at later time points will catch up.

The crosslinking of the BCR with the CR2/CD19/TAPA-1 co-receptor complex has been shown to decrease the threshold for B cell activation (170). It is possible that this mechanism is also involved in IgG3-mediated enhancement. However, as FTY720-treated mice present much less Ag inside follicles and FOB cells bind to IgG3-ICs very poorly, this mechanism seems unlikely to play a major role.
Summary

The ability of Abs to feedback regulate the Ab response has fascinated immunologists for decades, and the mechanisms behind this phenomenon have been difficult to identify. I believe that the data presented in this thesis, together with other recent observations, strongly suggest that interference in the accessibility of Ag to B cells is an important explanation for Ab feedback regulation (Fig. 6).

IgG-mediated suppression of Ab responses was believed to be the result of central B cell inhibition due to the co-crosslinking of the BCR and FcγRIIB by IgG-Ag ICs. However, suppression operates efficiently in mice lacking both FcγRIIB and other IgG Fc-receptors. The data presented here (paper I) provide clear evidence for epitope masking, as the suppression of IgG responses was strictly epitope specific.

IgE-mediated enhancement of Ab responses seem to be a consequence of the transport of IgE-complexed Ag by recirculating B cells into follicles, which is achieved through the binding of Ag to CD23. This can be achieved both when Ag is directly conjugated to an anti-CD23 Ab (paper II) or when it is bound to CD23 via IgE (paper III). Thus, the mechanism of action of IgE in feedback enhancement is simply to increase the effective concentration of Ag in follicles. Unlike what has been shown in vitro, the CD23⁺ B cells do not present IgE-Ag to T cells; rather, this is completed by a subset of DCs, CD8α⁺ cDCs (paper III).

IgG3/C-mediated enhancement of Ab responses is most likely caused by the capture of IgG3-ICs by MZB cells expressing CR1/2 followed by the transport of the Ag into follicles (paper IV). In follicles, the IC binds to FDC, which also express CR1/2. In bone marrow chimera experiments, the expression of CR1/2 on both B cells and FDC was required for optimal Ab responses. It cannot be excluded that the co-crosslinking of the BCR and CR1/2 on FOB cells plays a role by facilitating B cell signaling. However, we find it more likely that the transport role of MZB cells explains the requirement for CR1/2 on B cells.
Figure 6. **Feedback regulation via modification of Ag accessibility to cognate B cells.**

**A.** IgG together with particulate Ag can inhibit cognate B cell interaction with Ag via epitope masking. **B.** IgE-complexed Ag can be transported to B cell follicles via CD23$^+$ non-cognate B cells. This will increase the availability of Ag to CD8$^+$ cDCs, which endocytose it and present it to CD4$^+$ T cells, which in turn help B cells produce Abs. In addition, the increased Ag concentration in the follicle increases the chance that cognate B cells will bind. **C.** IgG3-complexed Ag can active C and then bind to MZB cells via CR1/2. MZB cells can then transport IgG3-Ag complexes into the follicle and increase the possibility that cognate B cells will interact with the Ag.
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