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Antibody Feedback Regulation

From Epitope Masking to T Helper Cell Activation

BY

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Abstract
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Antibodies have the ability to influence the antibody response against the very antigen they are specific for, in a process called antibody feedback regulation. Depending on the nature of the antigen, the antibody response can be either enhanced or almost completely inhibited. This thesis focuses on the underlying mechanisms of antibody feedback regulation *in vivo*.

Antigen-specific IgG can inhibit the antibody response to a particulate antigen. Based on its ability to inhibit B cell activation, the inhibitory FcγRIIB (low affinity receptor for IgG) has been suggested to be involved. Here we show that although FcγRIIB is required for efficient suppression *in vitro,* it is not required *in vivo*. Therefore, even though FcγRIIB can inhibit antibody responses, other mechanisms (such as epitope masking and enhanced antigen clearance) play a more dominant role *in vivo*.

The antibody response to soluble antigen is greatly enhanced when it is introduced to the immune system in complex with antigen-specific IgG or IgE. We found that FcγRIIB attenuates the magnitude of IgG-mediated enhancement. In mice lacking FcγRIIB, IgG enhanced the antibody response much more efficiently than in normal mice.

Since B cells require $CD4^+$ T cell help in order to become antibody-producing cells, we examined the $CD4^+$ T cell response to immune complexes *in vivo*. Using an adoptive transfer strategy with transgenic ovalbumin (OVA)-specific $CD4^+$T cells, we could show that the enhanced OVA-specific IgG response to IgG2a/OVA and IgE/OVA complexes was preceded by a potent OVA-specific $CD4^+$ T cell response. IgG2a-mediated enhancement was dependent on activating Fcγ receptors, whereas IgE-mediated enhancement was dependent on CD23, the low affinity receptor for IgE. We identified $CD23^+$ B cells as the responsible effector cells for IgE-mediated enhancement *in vivo*. Taken together, these results show that Fc receptor-mediated antigen presentation is a major mechanism underlying antibody feedback enhancement.

Keywords: Immune regulation, B cells, T cells, Antibodies, Fc Receptors, Antigen presentation, Transgenic/Knockout Mice

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To my family

luctor et emergo

List of Papers

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

I Karlsson MC, Getahun A, Heyman B.
FcγRIIB in IgG-mediated suppression of antibody responses: different impact *in vivo* and *in vitro*.
J. Immunol. 2001, 167(10):5558-5564

II Getahun A, Dahlström J, Wernersson S, Heyman B.
IgG2a-mediated enhancement of antibody and T cell responses and its relation to inhibitory and activating Fcγ receptors.
J Immunol. 2004, 172(9):5269-5276

III Getahun A, Hjelm F, Heyman B.
IgE enhances antibody responses and T cell activation *in vivo*. A central role for B cells.
Manuscript

Contents

Abbreviations

Ab	antibody
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
APC	antigen-presenting cell
B cell	B lymphocyte
BCR	B cell receptor
BM-DC	bone marrow-derived dendritic cell
BSA	bovine serum albumin
CD	cluster of differentiation
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FcR	Fc receptor
FcRγ	common γ-chain
FcRn	neonatal Fc receptor
FDC	follicular dendritic cell
HRBC	horse red blood cell
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
i.v.	intravenous
KLH	Keyhole limpet hemacyanin
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
OVA	ovalbumin
PFC	plaque-forming cell assay
RhD	rhesus D antigen
SHIP	SH_2-containing inositol polyphosphate 5-phosphatase
SRBC	sheep red blood cell
T cell	T lymphocyte
TCR	T cell receptor
Th cell	T helper cell
TNP	2,4,6-trinitrophenyl

Introduction

Our immune system is composed of a complex network of specialized cells and soluble mediators, which protects us against potentially pathogenic organisms such as viruses and bacteria. The immune system can be roughly divided into two parts, the innate and the adaptive immune system.

The innate immune response is a first line defense. It reacts quickly by recognizing molecular patterns characteristic for potential pathogens, such as viral DNA, RNA and bacterial cell-wall components. The induced response is appropriate for a certain group of pathogens but unspecific for the exact pathogen and has no memory. On the other hand, the adaptive immune response takes time to get started but is antigen specific and has a memory. This enables the immune system to respond quickly and specifically, by repeated exposure to the same pathogen. The components of the innate and adaptive immune system interact at various levels to ensure an appropriate and optimal immune response.

The immune system as a whole is tightly regulated. Given its destructive potential it is very important that an adequate response is initiated and eventually terminated in a controlled manner. Disturbances in the regulation could result in undesired responses, such as hypersensitivity and autoimmunity.

This thesis deals with antibodies, important soluble components of the adaptive immune response. I will focus on the ability of two classes of antibodies, IgG and IgE, to regulate the antibody response against the antigen for which they are specific, in a process called antibody feedback regulation. The outcome of antibody feedback regulation can either be an almost completely inhibited antibody response or a strongly enhanced antibody response, depending mainly on the nature of the antigen. The emphasis in the presented data will lie on the mechanisms underlying antibody feedback regulation *in vivo*. In addition the possible roles of antibody feedback regulation in the physiological and pathological immune response will be considered.

Background

Murine antibodies: structure and function

Antibodies (Abs), or immunoglobulins, are glycoproteins that can be found on the cell surface of B-lymphocytes, where they form the B cell receptor (BCR), or in a soluble form in serum and tissue fluids. An Ab is composed of two identical antigen-binding parts ($F(ab)_2$) and an Fc-domain which determines the isotype of the antibody. In mice the following isotypes can be found: IgM, IgD, IgG1, IgG2a, IgG2b, IgG3, IgA and IgE. The physiological function of Abs is to neutralize and eliminate the antigen (Ag) that induced their formation.

Elimination of Ag requires several effector mechanisms mediated by effector cells or the complement system. The Fc domain of the Ab interacts with Fc receptors (FcR) on the cell surface of these effector cells. The outcome of this interaction is dependent on the isotype of the Ab, the type of Fc receptor and effector cell type. Effector functions are e.g. enhanced phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC) and immediate hypersensitivity. Ab/Ag complexes can also enhance Ag presentation and some isotypes (IgM, Ig2a, IgG2b and IgG3) can activate the complement system via the classical pathway. Complement deposition on the Ab/Ag complex also allows effector functions mediated via complement receptors or direct lysis of the target cell via the membrane attack complex.

The antibody response.

The ingredients for an efficient Ab response, beside Ag, are B cells, T cells, antigen presenting cells (APCs), follicular dendritic cells (FDCs) and soluble mediators like complement and cytokines.

Upon binding of the Ag with its BCR, the B cell receives an initial activating signal. The B cell responds by initiating the cell cycle, but requires additional signals to start proliferating and differentiating into Ab-forming cells. These additional signals are provided by $CD4^+$ T cells, so called T helper cells. An exception is formed by non-protein Ags, like

polysaccharides, which can directly activate B cells and are called T-independent Ags.

T cells recognize Ag in the form of peptides presented on MHC molecules. Ag is taken up by APC, such as macrophages and dendritic cells (DCs), and is subsequently processed and presented on MHC class II molecules. Ag-specific $CD4^+$ T cells will be activated upon interaction with the MHC/peptide complex and additional costimulatory molecules present on the APC. B cells are also APCs, although their ability to activate naive T cells remains a matter of debate. Upon binding to Ag with the BCR, the B cell will internalize the Ag, process it and present it on MHC class II molecules. This enables the activated Ag-specific T cell to interact with the B cell, and provide the additional stimuli promoting B cell proliferation and differentiation. T helper cells release cytokines and provide stimuli by cell-cell interactions. The B cell differentiates into an Ab-forming cell, initially producing IgM. Under the influence of cytokines, class-switching to produce other Ab isotypes, takes place. Depending on the cytokines T helper (Th) cells produces, they can be divided into Th1 and Th2 cells. Th1 cells mainly produce IFNγ and IL-2, stimulating class-switching to IgG2a, whereas Th2 cells produce IL-4 and IL-5, which promotes the production of IgG1 and IgE.

Some cells of the expanded B and T cell populations will migrate into follicles, structures in secondary lymphoid organs, to form germinal centers (1). FDCs that nested within the germinal center retain immune complexes on their cell surfaces by binding them to their Fc and complement receptors. Within the germinal center, the B cells will go through a cycle of somatic hypermutation, proliferation and selection for clones with the highest binding affinity. This process is called affinity maturation. Ag trapped in immune complexes on the FDC is thought to play an important role in the high-affinity selection process. Some of the high-affinity B cells will exit the germinal center and differentiate into plasma cells or into memory B cells. Plasma cells produce Abs and can be found in secondary lymphoid organs and bone marrow. The memory B and T cells stay dormant until the next time the Ag appears. Then memory cells initiate a secondary response, which is characterized by a rapid onset, high Ab titers and high affinity Abs.

Fc receptors

As mentioned above, a large part of the effector functions of antibodies is mediated by interactions with Fc receptors. Fc receptors exist for every antibody class: FcγRs bind IgG, FcαR binds IgA, FcεRs bind IgE, FcμRs

bind IgM and FcδR binds IgD (reviewed in (2)). Below, I will focus on the Fc receptors for IgG and IgE, in particular FcγRIIB and CD23.

Fc receptors for IgG

In mice there are four Fc receptors for IgG: FcγRI (CD64), FcγRIIB (CD32) and FcγRIII (CD16) and the neonatal FcR (FcRn). FcγRI and FcγRIII are activating receptors, which initiate effector functions upon crosslinking, whereas FcγRIIB can inhibit this activation upon co-crosslinking (3, 4). FcγRI is a high affinity receptor, capable of binding monomeric IgG2a and complexed IgG. It is expressed on macrophages, monocytes and DCs (2, 5). FcγRIII is a low affinity receptor, binding only complexed IgG. It is expressed on macrophages, mast cells, DCs and NK cells (2). FcγRI and FcγRIII are hetro-oligomeric complexes, in which the respective ligand-binding α chains are associated with the common γ chain (FcRγ). FcγRIII is also associated with a β-chain. FcRγ is required for the assembly and for the triggering of their various effector functions. It contains an immunoreceptor tyrosine-based activation motif (ITAM), which gets phosphorylated upon receptor crosslinking. Sequential activation of *scr* and *syk* family tyrosine kinases result in an activating signaling cascade (3).

Analysis of activating Fcγ receptor-deficient mice confirmed the importance of FcγRs in mediating IgG effector functions and in the development of inflammatory disease (reviewed in (4, 6, 7)). Cells from FcRγ-deficient mice, lacking expression of FcγRI and FcγRIII, are unable to facilitate IgG-mediated effector functions such as phagocytosis and ADCC (8). The individual roles for FcγRI (9, 10) and FcγRIII (11) have been studied with cells from mice deficient for the specific receptors. In general, involvement is dependent on the IgG isotype (FcγRI mainly for IgG2a and FcγRIII for IgG1) and the cell type involved (FcγRI for processes involving mononuclear cells and FcγRIII for other cell types). FcRγ$^{-/-}$ mice are unable to develop IgG-triggered inflammatory responses (8, 12, 13) and are protected against IgG-dependent inflammatory diseases (14-18). Further analysis of mice specifically lacking either FcγRI, FcγRIII, or both, revealed that in some disease models FcγRIII was predominantly responsible for inflammatory responses (11, 18-20). In other models both receptors were involved (10, 21, 22), albeit sometimes at different disease stages (23).

FcγRIIB is a low affinity IgG receptor, composed of a single chain, which exists in several isoforms. FcγRIIB is widely expressed on hematopoietic cells, missing only from erythrocytes, T and NK cells (2). FcγRIIB1 is an isoform that cannot be internalized and is the only FcγR present on B cells. The other main isoform, FcγRIIB2, can be internalized and is mainly present on macrophages (2).

FcγRIIB has an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. When, by co-crosslinking, the ITIM motif comes in the vicinity of an ITAM bearing receptor-complex, the tyrosine in the ITIM motif will be phosphorylated. This phosphorylation generates an SH2 recognition domain that serves as a binding site for the inhibitory signaling molecule SHIP (24, 25). Besides SHIP, FcγRIIB on activated B cells can also use SHIP2 (26). Recruitment of SHIP leads to downstream events interfering with the activating signaling. ITAMs are present in a large number of immunological receptors such as FcRs, the BCR and the T cell receptor (TCR). The ITIM motif present in FcγRIIB can inhibit the signaling of all the above mentioned ITAM-bearing receptors (27). At least one type of inhibitory activity by FcγRIIB is independent of the ITIM motif. Upon homo-aggregation of FcγRIIB on B cells a proapoptotic signal is generated, which has been suggested to be involved in the negative selection of low affinity B cells in the germinal center (28).

FcγRIIB can negatively regulate many processes. It has been shown to negatively influence activation of B cells (24, 29-31), T cells (27, 32) and mast cells (27, 33), as well as Ag presentation (34, 35) and phagocytosis (36) *in vitro*. FcγRIIB-deficient mice have augmented Ab responses and anaphylactic responses (37). These mice also display enhanced autoimmunity, both induced (17, 23, 38, 39) and spontaneous (40, 41), and augmented IgG-mediated inflammation (16, 42-44).

FcRn consists of a MHC class I related α chain associated with β_2 microglobulin. It is expressed on human and murine vascular endothelial cells, intestinal epithelial cells and human placenta and it binds to all subclasses of IgG (45). FcRn transports maternal IgG over the human placenta and the murine neonatal intestine. It also protects serum IgG from degradation (46). Recently it has been shown to transport IgG across the intestinal barrier into the lumen and as IgG/Ag complex back into the lamina propria, where the IgG/Ag complex is processed by DCs (47). A similar function for FcRn has been suggested in the lung (48). In addition, in humans FcRn is expressed in monocytes, macrophages and dendritic cells and binds IgG, suggesting a major role for this receptor within immunity (49).

Finally it is not impossible that new FcRs for IgG will be discovered. Recently new Fcγ receptor-related genes with a preferential B cell expression have been identified (50, 51). It is not known yet if they bind IgG, but they contain ITIM and ITAM-like motifs, implying a regulatory function.

Fc receptors for IgE

There are two FcRs for IgE, the high affinity FcεRI and the low affinity FcεRII (CD23) (reviewed in (52-54)). In mice, only one isoform of FcεRI has been described. This is a tetramer consisting of one α chain, one β chain and two disulfide-linked FcRγ chains. FcεRI is expressed in non-B non-T cells, including mast cells and basophiles and upon crosslinking induces degranulation of these cells.

Unlike other Fc receptors, CD23 does not belong to the immunoglobulin superfamily, but is instead a C-type lectin (55). It consists of a C-terminal lectin domain, which is the binding site for IgE, followed by a stalk region, a transmembrane region and a very short cytoplasmic domain. The receptor is present on the cell surface both as monomers and trimers, the latter having a higher affinity for IgE (56). Membrane bound CD23 is cleaved by an unknown protease at several sites within the stalk region. All soluble fragments, except one, can bind IgE, but only with low affinity.

In humans, CD23 is expressed in two isoforms, CD23a and CD23b (57). CD23a is constitutively expressed on B cells; CD23b is expressed on $CD5^+$ B cells, Langerhans cells, bone marrow-derived mast cells, monocytes, eosinophils, platelets and some T cells. Upon IL-4-stimulation, CD23b is upregulated on B cells and Langerhans cells ((54, 57) and references therein). Mice constitutively express a homologue of CD23a on B cells (58) and FDCs (59). Upon stimulation with IL-4 and lipopolysaccharide, CD23b mRNA can be detected in murine B cells and other haematopoietic cells (60). Under certain culture conditions murine Langerhans cells also express CD23 (61).

Several roles have been suggested for CD23 in the immune response. Its presence on FDCs has been implied to play a major role in the trapping and retaining of IgE immune complexes (59). *In vitro* experiments have shown that CD23 expressed on B cells mediates efficient uptake of Ag in complex with IgE, followed by efficient presentation of antigenic peptides to T cells (62-65). In human B cell lines, CD23 is non-covalently associated with MHC class II molecules. Upon IgE/Ag binding, these complexes are endocytosed, shuttled to intracellular compartments associated with MHC class II loading and recycled to the cell surface, suggesting a role for this association in antigen presentation (66).

Regulation of the B cell response has also been suggested, as co-crosslinking of CD23 with the BCR induces apoptosis of splenic B cells (67) and prevents B cells from proliferating and differentiating (68). Cross-linking of CD23 alone also inhibits proliferation and differentiation of B cells (69). In

humans, CD23 was also found to interact with CD21 and CD11b/CD11c, influencing B cell activation and cytokine release from macrophages, respectively (70, 71). In mice only the interaction with CD11b/CD11c on macrophages has been confirmed (72). The function of soluble CD23 is unclear. On human B cells soluble CD23 can act as an autocrine growth factor (73), whereas its function in mice is unknown (74).

Insight into the role of CD23 *in vivo* came from CD23-mutant mice. Mice lacking CD23, due to gene targeting, have no detectable defects in lymphocyte differentiation and have generally normal Ab responses (75, 76). However on two accounts the Ab response differs in $CD23^{-/-}$ mice compared to Ab response in wildtype mice. 1) $CD23^{-/-}$ mice cannot enhance the Ab response to IgE/Ag complexes (76) (to be described in detail below). 2) Elevated (Ag-specific and total) IgE levels were detected in $CD23^{-/-}$ mice after immunization with Ag in alum (75).

A role for CD23 in suppressing IgE levels was confirmed in transgenic mice overexpressing CD23 (77, 78). The negative effect on IgE production is not associated with expression of CD23 on B cells, but instead with its presence on FDCs (79). So CD23 seems to have a dual role in controlling Ab responses, enhancing Ab responses to IgE/Ag complexes and negatively regulating the Ab response to Ag in alum. This difference could be explained by the action of the adjuvant, inducing inflammation and expression of CD23b on a non-B cell, or the involvement of different CD23 ligands. In the case of IgE/Ag complexes there is a direct interaction between CD23 and IgE, whereas after immunization with Ag in alum other ligands such as CD21 or CD11b/CD11c, could be involved.

Finally in allergic rats (80) and mice (81) an increased intestinal transepithelial Ag transport has been observed which was mediated by IgE and CD23.

Feedback regulation by antibodies

Abs have the ability to regulate the Ab response to the Ag they are specific for, in a process called Ab feedback regulation (reviewed in (82, 83)). The Ab response can be greatly enhanced or efficiently suppressed during feedback regulation. Factors determining the outcome of feedback regulation are the Ab class, the nature of the Ag, Ab affinity, administration route, the ratio Ag:Ab and the temporal space between administration of Ag and Ab.

In general, Ab responses to particulate Ags can be suppressed, although suppression of Ab responses to soluble Ags as tetanus toxoid and keyhole

limpet hemocyanine (KLH) has also been reported. All IgG subclasses (84-86) as well as IgM (87) and IgE (88, 89) are able to suppress Ab responses. Suppression often Ag specific (i.e. an Ab to a certain epitope suppressed the Ab response to the whole Ag) (85, 86, 88). However epitope-specific suppression also has been observed (90, 91).

Enhancement of Ab responses is Ag-specific but not epitope specific. All IgG subclasses (92-95) and IgE (76, 96) can enhance the Ab response to soluble protein Ags. IgM (84, 97) enhances Ab responses to particulate Ags and large soluble protein Ags. Since IgM is dependent on complement to enhance (98, 99), it probably requires to bind a larger Ag in order to undergo the conformational changes required to activate complement.

In this thesis I have studied the mechanisms underlying IgG-mediated suppression, IgE-mediated enhancement and IgG-mediated enhancement. Below I will describe the current view on the underlying mechanisms of these forms of feedback regulation.

IgG-mediated suppression

IgG can efficiently suppress (>99%) the Ab response to particulate Ags (reviewed in (82, 83, 100)). A clinical application can be found in Rhesus prophylaxis, where pregnant women which lack the rhesus D antigen (RhD) on their erythrocytes receive IgG anti-RhD to prevent them from initiating an immune reaction against fetal RhD^+ erythrocytes transferred via trans-placental hemorrhage (101).

IgG can suppress both a primary and a secondary Ab response (82). Priming of T cells occurs (88) implying that suppression takes place at the level of antigen-B cell interaction. Suppression does not seem to directly affect the B cells, as B cells from a suppressed animal are capable of initiating a normal Ab response to the Ag after transfer to irradiated animals (102).

Studies on the importance of the suppressing antibody's Fc-domain have generated conflicting data. The suppressive effect of $F(ab')_2$ fragments was compared to intact Ab and has been reported to induce equally efficient (88, 103, 104) as well as less efficient suppression (85, 92, 105, 106) *in vivo*. Complement is not required, since mutated Abs, which are unable to activate complement, suppress efficiently (107).

Three different mechanisms have been suggested. The first mechanism is epitope masking, in which the administered Abs sterically hinder interaction with the BCR, thereby preventing B cell activation. Secondly, efficient elimination of IgG/Ag complexes by ($Fc\gamma R^+$) phagocytic cells could also

prevent B cell activation. Finally, co-crosslinking of the BCR with FcγRIIB by the IgG/Ag complex could inhibit B cell activation. The latter two mechanisms are dependent on the presence of FcγRs. Recently our group showed that IgG can efficiently suppress the Ab response in mice lacking the known Fc receptors for IgG (FcγRI, II, III and FcRn) (88). These results showed that the ability of IgG to suppress is independent of the Fc part of the Ab and suggest that the predominant mechanism behind feedback suppression is epitope masking.

IgG-mediated enhancement

Besides being able to suppress Ab responses, IgG can also strongly enhance Ab responses to soluble protein Ag (reviewed in (83, 108)). Both primary IgM (109) and IgG (92-94) responses are enhanced. IgG immune complexes are also potent induces of immunological memory (110), giving early rise to high affinity B memory cells (111).

All murine IgG subclasses can enhance Ab responses. With the exception of IgG3 (95), which will not be further discussed, all IgG subclasses enhance in the absence of a functioning complement system (99, 112). Instead IgG1, IgG2a and to a lesser extent IgG2b are dependent on the presence of activating Fc receptors to induce Ab enhancement. Mice lacking FcγRI and FcγRIII (due to FcRγ deficiency) do not show IgG-mediated enhancement (94). Since mice only lacking FcγRIII respond normally, a major role for FcγRI in IgG-mediated enhancement is implied (94). Activating FcγRs are expressed on a number of cell types, macrophages, DCs and FDCs being the most relevant for Ab responses. Bone marrow transfer experiments showed that FDC do not need to express Fc receptors for efficient IgG-mediated enhancement to occur (110). Therefore, the most likely mechanism underlying IgG-mediated enhancement is enhanced Fc receptor-mediated Ag uptake and presentation by dendritic cells or macrophages. The enhanced T cell priming would then enable better T cell help to Ag-specific B cells.

IgE-mediated enhancement

Immunization with IgE immune complexes enhances the Ab response, upregulating primary IgM, IgG and IgE responses (76, 96, 113, 114). A rapid increase in specific IgG-producing B cells is seen, peaking 6 days after primary immunization (114). Serum titers of specific IgG and IgE peak at day 7 and remain high for weeks (114). In addition, priming with IgE immune complexes gives a more efficient immunological memory (113).

IgE-mediated enhancement is completely dependent on CD23, as IgE cannot enhance in mice treated with anti-CD23 mAbs (96, 113) or in CD23-

deficient mice (76, 115). Lack of involvement of FcεRI was confirmed in FcRγ-deficient mice, which have no surface expression of FcεRI (94). As CD23 is only constitutively expressed on B cells (58) and FDC (59), one or both of these cells types should be involved. Involvement of the IL-4-inducible CD23b (expressed on other hematopoietic cells) is unlikely as IgE-mediated enhancement is unperturbed in mice lacking IL-4, suggesting that constitutively expressed CD23a is sufficient (116).

The presence of CD23 on FDCs has been implied to play a major role in the trapping and retaining of IgE immune complexes (59). Efficient trapping and increasing of the effective Ag concentration in the germinal center is a possible mechanism behind IgE-mediated enhancement. However, using an adoptive transfer system, it was shown that in order for IgE-immune complexes to enhance Ab responses, bone marrow derived cells (B cells), but not FDC, need to express CD23 (115).

The necessity for B cells to express CD23 suggests two possible mechanisms, which are not mutually exclusive. One possibility is that co-crosslinking of CD23 with the B cell receptor could initiate a signaling cascade that directly activates the B cell in a similar way as IgM immune complexes are thought to do via CD21/CD19 (99). However, this is not very likely since co-crosslinking of CD23 and the BCR can have a negative effect on B cell activation (68). A more likely scenario, is that CD23 on B cells mediates efficient uptake and subsequent presentation of Ag present in IgE immune complexes. This has been shown to occur *in vitro* (62-65). This would result in a more efficient activation of Ag-specific T helper cells, which in their turn can stimulate the Ab response.

Immune complex mediated antigen presentation on MHC class II

MHC class II presentation and $CD4^+$ T cell activation

Three types of APCs are equipped for MHC class II antigen presentation: B cells, macrophages and dendritic cells. APCs acquire exogenous Ag via (macro)pinocytosis or receptor-mediated endocytosis (e.g. via FcRs, mannose receptors, BCR). Once internalized, protein antigens become localized in intracellular vesicles called endosomes, which have an acidic pH and contain proteolytic enzymes. Endosomes interact with lysozomes and the internalized proteins are enzymatically degraded into 10 to 30 aa long peptides. Meanwhile, MHC class II molecules are synthesized in the ER and assembled together with the invariant chain (Ii), which shields the peptide-binding cleft. Exocytic vesicles from the ER, containing the newly

synthesized MHC class II molecules, fuse with the endosome, where Ii is degraded leaving a 24 aa remnant peptide named CLIP bound in the peptide binding cleft. A non-classical MHC class II molecule called H-2M exchanges CLIP for the antigenic peptide. The bound peptide stabilizes the MHC class II molecules and the stabilized class II molecules are delivered to the cell surface. For successful T cell priming (i.e. activation) the APC also needs to upregulate the levels of costimulatory molecules. Often the receptor-mediated endocytosis (e.g. crosslinking of FcR or BCR on DCs and B cells, respectively) results in activating signals, which ultimately result in an improved ability to prime T cells. Also other environmental factors that stimulate cytokine receptors or pattern recognition receptors can have these effects of APCs.

In general, $CD4^+$ T cells are specific for peptides derived from extracellular proteins. In order to become activated the T cell requires two signals. First, the Ag-specific TCR needs to recognize the peptide it is specific for in the context of a MHC class II molecule on an APC. Its co-receptor, CD4, also needs to interact with a specific region on the MHC class II molecule. While adhesion molecules, such as LFA-1, stabilize the APC-T cell interaction, costimulatory receptors on the T cell, such as CD28, interact with costimulators, such as B7-1/B7-2, on the APC, to receive the second signal, resulting in T cell activation. This activation will lead to a clonal expansion of the activated T cell and subsequent differentiation into an effector or memory cell.

IgG-mediated antigen presentation

All three FcγR subtypes mediate Ag presentation (reviewed in (117)). The relative contribution of each type is most likely dependent on which IgG subclasses are incorporated in the immune complexes (ICs) as well as on the overall size of the complex. Bone marrow-derived macrophages present human IgG ("monovalent ICs") mainly via FcγRI (9) while only multivalent ICs were presented via FcγRIII (118).

Comparison between murine FcγRIIB2- and FcγRIII-mediated Ag presentation revealed that the associated FcRγ chain enables FcγRIII to present a broader spectrum of epitopes than FcγRIIB2, owing to different intracellular targeting (119, 120). Studies with human FcγRI show that although the FcγRI α chain itself can mediated Ag presentation, the presence of functional FcRγ chains increase the efficiency of Ag presentation, at least in part by influencing intracellular targeting (121). The ability of FcγRIIB1 to facilitate IgG-mediated antigen presentation is a matter of debate. Some studies claim it cannot (31), while others show that B cells expressing

FcγRIIB1 can present IgG/Ag-complexes in a FcR-dependent manner, depending on which Ag is present the immune complex (122, 123).

In vitro, human and murine FcγR$^+$ APCs present IgG-complexed Ag at concentrations over a 100-fold lower than uncomplexed Ag. This has been demonstrated for murine bone marrow-derived dendritic cells (BM-DCs) (124-126), macrophages (9, 127), Langerhans cells (128), and B cells (122, 123) as well as for human peripheral blood DCs (129, 130), monocytes (130), macrophages (127, 131), and B cells (132). Crosslinking of activating FcγRs by IgG/Ag complexes also induce DC maturation, turning DCs into better APCs (5, 124, 125, 133). Furthermore, FcγRIIB negatively influences this IC-mediated maturation (134), suggesting that the balance in expression between activating and inhibitory FcγRs can influence the ability of DCs to present IgG/Ag complexes.

Recently a number of studies have suggested that IgG also mediates efficient FcγR-dependent Ag presentation *in vivo*. Delayed-type hypersensitivity (DTH) reactions, mediated by CD4$^+$ Th1 cells, were decreased in FcRγ$^{-/-}$ mice suggesting a role for IgG/Ag complexes in T cell priming (135). Studies in which mice were immunized with IgG/Ag complex-pulsed BM-DCs showed that these mice have an enhanced *ex vivo* T cell response (124) and improved CD4$^+$ effector functions *in vivo* such as enhanced antibody responses (124, 125, 136, 137), stronger DTH reaction (125) and better tumor immunity (124, 125). Whether IgG/Ag complexes induce a Th1- or Th2-polarized response is not clear. Some studies support a Th1-polarized response (124, 125) others a Th2-polarized T cell response (137, 138).

IgE-mediated antigen presentation.

IgE-mediated Ag presentation is less well studied than IgG. The ability of FcεRI to mediate effective Ag presentation of IgE bound Ag has been demonstrated *in vitro* for isolated human monocytes (139) and peripheral blood DCs (140). Further studies on human BM-DCs revealed that FcεRI efficiently channels Ag into MHC class II compartment-like organelles (141). The expression of FcεRI is more restricted in the mouse: in this species only bone marrow-derived mast cells have been shown to present Ag taken up via this receptor (142).

CD23 is involved in antigen presentation both in human and murine systems. *In vitro* experiments with human EBV-transformed B cells (63-65), a murine B-cell hybridoma (143), and murine spleen cells (62) all demonstrate that B cells present IgE-complexed Ag in a CD23-dependent manner at concentrations a 100- to a 1000-fold lower than they present uncomplexed Ag. Functional studies using the human CD23 isoforms showed that only

CD23a facilitates IgE-mediated endocytosis (144), implying an important role for B cells.

The occurrence of CD23-mediated Ag presentation *in vivo* has been suggested by experiments in which Ag covalently coupled to an anti-CD23 mAb was used. *In vitro* these conjugates are very effectively presented and after immunization in mice they induce enhanced Ag-specific IgG and IgE levels (145).

Present investigation

Aims

The ability of Abs to regulate the Ab response against the Ag for which it is specific is well described. However, the precise mechanism by which this Ab feedback regulation occurs *in vivo* still remains elusive. This thesis is based on three papers, dealing with different branches of feedback regulation. Using mouse models for IgG-mediated suppression, IgG2a-mediated enhancement and IgE-mediated enhancement, I have tried to answer the following questions:

- Is FcγRIIB involved in IgG-mediated suppression of Ab responses? (Paper I)
- How does FcγRIIB affect the Ab response to IgG-immune complexes? (Paper I & II)
- Do IgG-immune complexes induce a T cell response *in vivo*? (Paper II)
- Do IgE-immune complexes induce a T cell response *in vivo,* and if so, are B cells the effector cells? (Paper III)

Experimental systems

Mice

To study the role of different Fc receptors in Ab feedback regulation we used a panel of gene-targeted mice (Table I) on different genetic backgrounds with the appropriate wild type controls (BALB/c and CBA/J). Mice of all MHC haplotypes respond to sheep red blood cells (SRBCs) and can therefore be used in IgG-mediated suppression experiments. However, mice with MHC class II molecules of the b haplotype are unable to facilitate IgG- and IgE-mediated enhancement (146, 147). Since most gene-targeted founder animals are on a mixed C57BL/6 x 129/Sv background (carrying H-2b) we have backcrossed mutant mice onto responder backgrounds (CBA/J and BALB/c) for 10 generations.

Table 1. Characteristics of the used Fc receptor-knockout mice. * Original reference describing the founder animals. § Genetic background onto which the founder animals were backcrossed for 10 generations. ¶ Number of the paper in which the mutant mice were used.

Mutant mice	Deficiency	Founder*	Background§	Paper¶
FcγRIIB$^{-/-}$	FcγRIIB	(37)	CBA	I, II
			BALB/c	II
FcRγ$^{-/-}$	FcγRI, FcγRIII, FcεRI	(8)	BALB/c	II, III
CD23$^{-/-}$	CD23	(76)	BALB/c	III
			CBA	III

For *in vivo* T cell activation studies (II & III), DO11.10 mice were used as a source of OVA-specific T cells. DO11.10 mice carry a construct containing rearranged TCRα and TCRβ genes encoding a TCR specific for ovalbumin (OVA) 323-339 bound to I-A^{d} class II molecules (148) and have been bred onto a BALB/c background for > 15 generations

Immunizations and assays for antibody responses

Immunization: In IgG-mediated suppression studies (I) the different experimental groups were immunized intravenously (*i.v.*) with SRBC and horse red blood cells (HRBC). The Ab response against SRBCs was suppressed by administering up to 10 μg anti-SRBC polyclonal IgG at the time of immunization or up to 5 days after immunization. The HRBC response served as a specificity control.

To study the ability of IgG to suppress a secondary response, mice were primed with SRBCs and the serum anti-SRBC titers were followed in time by enzyme-linked immunosorbent assay (ELISA). After > 79 days the serum levels had almost returned to background levels. The mice were then directly immunized or, to avoid interference of endogenous anti-SRBC Abs, primed spleen cells were transferred to irradiated (600 rad) syngeneic recipients. Twenty-four hours after transfer the recipients were immunized *i.v.* with SRBCs ± anti-SRBC IgG.

The ability of IgG to suppress the Ab response was also measured *in vitro*. Mice were primed with SRBCs intraperitoneally. After 4 days the spleens were collected and single cell suspensions were prepared. The primed spleen cells were cultured with SRBCs and HRBCs in the presence or absence of anti-SRBC polyclonal IgG for 4 days.

To study Ab-mediated enhancement, mice were immunized i.v. with Ag alone or with preformed Ag/Ab complexes, in physiological salt solutions (II-III). As Ag, 2,4,6-trinitrophenyl (TNP)-conjugated bovine serum albumin (BSA) and OVA was used with uncoupled OVA or KLH as specificity controls, respectively. Monoclonal IgG2a anti-TNP (93) and IgE anti-TNP (149) were used to form immune complexes. In paper III, IgE was administered 1 hour before Ag instead of as preformed Ag/Ab complexes. For studies of the secondary response after priming with IgG/BSA complexes, mice were boosted with BSA in PBS subcutaneous in the flank.

Assays: Serum Ab responses were quantified by ELISA. Total Ag-specific IgG levels were measured by BSA-, OVA- or KLH-specific ELISA. Since the injected Abs are specific for TNP, they did not interfere. The number of Ab forming cells producing SRBC- or HRBC- specific IgM were quantified by Jerne's hemolytic plaque forming cell (PFC) assay. The number of Ab-forming cells producing BSA-, SRBC- or HRBC-specific IgG were quantified using specific enzyme-linked immunospot (ELISPOT) assays. On a macroscopic level, the Ab response was also examined by determining the number of germinal center after immunization with IgG2a/Ag complexes by immunohistochemistry.

Statistical analyzes: Statistical differences between groups were calculated by Student's t-test, with the exception of the *in vitro* suppression experiments for which a Wilcoxon rank sum test was used. p values are presented as not significant, $p>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $p<0.001$. The degree of suppression or enhancement was calculated as the geometrical mean of the experimental group (Ag + Ab) divided by the geometrical mean of the control group (Ag alone).

Assays for T cell responses

Delayed type hypersensitivity (DTH) reactions were provoked as an indication for T cell activation (II), as DTH is induced by Th1 cells (150). Mice were primed with 10 or 100 μg of BSA emulsified in complete Freund's adjuvant intradermally at the root of the tail. After a week the mice were challenged with 10 μg BSA in PBS in the right ear and PBS alone in the left ear. The difference in swelling between the two ears was measured in time.

To directly study T cell activation *in vivo* (II & III) an adoptive transfer system developed in Dr. Marc Jenkins group was used (151). DO11.10 spleen cells were adoptively transferred *i.v.* into unirradiated BALB/c mice. The fate of the transgenic OVA-specific T cells could then be followed by flow cytometry by means of a clonotypic mAb, KJ1-26, specific for the

DO11.10 TCR. Multicolor flow cytometry enabled us to follow both changes in population size after immunization with Ag/Ab complexes as well as phenotypic changes of the Ag-specific T cells.

To avoid transfer of $CD23^{+}$ or $FcR\gamma^{+}$ cells in experiments with $CD23^{-/-}$ BALB/c, $Fc\gamma RIIB^{-/-}$ or $FcR\gamma^{-/-}$ recipients, only magnetically isolated $CD4^{+}$ DO11.10 cells were transferred in such experiments (II & III). In some experiments CD19 expressing cells were magnetically depleted before adoptive transfer. As a second approach to study the involvement of B cells, "untouched" B cells from wildtype mice were isolated with anti-CD43-conjugated magnetic beads and co-transferred with purified $CD4^{+}$ DO11.10 cells into $CD23^{-/-}$ BALB/c mice.

Results & Discussion

IgG mediated suppression in the absence of FcγRIIB (Paper I)

Recently we showed that IgG can efficiently suppress the Ab response in mice lacking the known Fc receptors for IgG (FcγRI, II, III and FcRn) (88). These results created some controversy (152, 153), in particular the apparent lack of a role for FcγRIIB in feedback suppression. Only suppression of the primary response was studied in $Fc\gamma RIIB^{-/-}$ mice, when SRBC and IgG anti-SRBC were administered either as preformed immune complexes or within one hour of each other. Here, we tested under more stringent conditions whether FcγRIIB is required for efficient suppression.

IgG efficiently suppresses Ab responses in $Fc\gamma RIIB^{-/-}$ mice under all tested conditions

It has been suggested that although epitope masking can prevent the initiation of an Ab response, suppression of an already established Ab response requires B cell inhibition by FcγRIIB (105). To test if FcγRIIB is required to suppress an already established Ab response we administered IgG to $Fc\gamma RIIB^{-/-}$ and wildtype mice up to 5 days after immunization with SRBC**.** In both strains the Ab response was equally efficiently suppressed when IgG was given up to 4 days after Ag. Interestingly, a delay of two days was observed between the moment the IgG was administered and efficient suppression (I). This observation suggests that the B cell needs continuous stimulation via the BCR in order to survive and/or produce Abs. Probably, the high affinity polyclonal anti-SRBC Abs successfully competes with the low affinity BCR, in a manner that has been proposed to occur during affinity maturation in the germinal center reaction (154). The observation that only high affinity Abs suppress efficiently support this (85, 155).

Next, we examined the ability of IgG to suppress a secondary response. Secondary responses are in general more difficult to suppress, probably due to the presence of high-affinity memory cells (82). Also, on activated B cells the expression of FcγIIB is upregulated (156) and therefore it might be easier to detect involvement of FcγRIIB during a secondary Ab response. Mice primed only with SRBC were re-challenged with SRBC or SRBC plus IgG anti-SRBC. The secondary IgG and IgM responses were equally efficiently suppressed in wildtype and FcγRIIB$^{-/-}$ mice. We obtained similar results with a slightly different approach, transferring primed spleen cells to irradiated syngeneic mice to prevent interference of endogenous anti-SRBC IgG (I).

In conclusion, IgG can induce efficient suppression of both the (ongoing) primary IgM response and the secondary IgM and IgG response in absence of FcγRIIB.

Impaired suppression of secondary Ab responses in vitro without FcγRIIB

Previously we made a number of observations, which suggested FcγR-dependence for IgG-mediated suppression *in vitro* (157, 158). To investigate whether FcγRIIB was involved in IgG-mediated suppression *in vitro*, spleen cells from primed FcγRIIB$^{-/-}$ and wildtype mice were incubated with SRBC in the presence or absence of IgG anti-SRBC. Spleen cells from FcγRIIB$^{-/-}$ were significantly impaired in the ability to induce efficient suppression compared to wildtype cells (I).

LPS-activated B cells express 10-fold higher numbers of FcγRIIB than resting B cells (156). To test if the use of primed B cells, with increased FcγRIIB expression, was the reason behind the observed difference between FcγRIIB requirement *in vivo* and *in vitro*, or if it was the *in vitro* culture conditions *per se*, identically primed spleen cells were adoptively transferred into irradiated syngeneic hosts. IgG-mediated suppression occurred to the same degree in FcγRIIB -/- mice as in wildtype controls, demonstrating that it was not the use of primed B cells but the *in vitro* culturing conditions that caused the differences (I).

FcγRIIB and the mechanism behind IgG-mediated suppression

Undeniably, FcγRIIB can negatively regulate Ab-mediated processes, as ADCC, antigen presentation and inflammatory mediator release *in vitro* (31, 33-36) and *in vivo* (16, 37, 42, 43). Mice lacking FcγRIIB also have stronger Ab responses to uncomplexed thymus dependent and independent Ags (37) as well as after immunization with immune complexes consisting of IgG and soluble protein Ags (94). These findings imply that FcγRIIB can indeed negatively influence the Ab response (see below), and an interesting question is why FcγRIIB is not required in IgG-mediated suppression.

In general the *in vivo* role of FcγRIIB seems to be that of a negative modulator, fine-tuning Ab-mediated reactions but not totally inhibiting them (4). With respect to Ab responses, FcγRIIB seems to function as a safety valve, setting an upper threshold for Ab levels.

Inhibition of B cell activation following co-crosslinking of FcγRIIB and the BCR has mostly been seen in *in vitro* experimental systems (24, 29-31). The fact that we observe a FcγRIIB-dependent suppression *in vitro* shows that FcγRIIB can inhibit Ag-specific B cells. Since FcγRIIB is dispensable *in vivo* the obvious explanation is that *in vivo* additional mechanisms exist that are more potent. One possibility is a form of efficient Ag clearance operating besides epitope masking. However the precise nature of this IgG-Ag clearance remains to be defined, since IgG-mediated suppression takes place in the absence of all known IgG-Fc receptors (88). In addition, complement-mediated clearance is also unlikely as IgG, unable to activate complement, also suppresses efficiently (107).

Support for a major role of epitope masking and immune complex clearance in IgG-mediated suppression *in vivo* can be found in the clinic. Pathogen-specific maternal antibodies present in the newborn interfere with the humoral response to vaccines (159). A number of observations made in the field of infant vaccination have striking parallels our IgG-mediated suppression model, most importantly: 1) T cell priming is unaffected in both cases (88, 160), implying that IgG/Ag complexes are taken up by phagocytes and presented to T cells. 2) The ratio between IgG and Ag determines in both cases the efficiency of suppression (86, 159). Although, theoretically Ag excess also would dilute FcγRIIB signaling, it is easier to envision how it would reduce the degree of epitope masking and thereby overcome suppression.

In conclusion, *in vivo* IgG mediated suppression functions efficiently in the absence of FcγRIIB under all conditions tested. Epitope masking and/or efficient Fc- and complement independent clearance of IgG/Ag complexes are the most likely mechanisms underlying IgG-mediated suppression.

IgG2a-mediated enhancement of antibody and T cell responses (Paper II)

Previous work by our group showed that the outcome of an Ab response to IgG2a/Ag complexes is dependent on the balance between activating and inhibitory Fc receptors. In mice lacking activating FcγRs, IgG/Ag complexes induce no enhancement, whereas in mice lacking the inhibitory FcγRIIB, IgG/Ag complexes induce a strongly increased Ab response (94). Here we

focus on the mechanisms by which these two types of FcγRs regulate the Ab response to IgG2a/Ag complexes.

IgG2a/Ag complexes enhance Ab and T cell responses in a FcγR-dependent manner.

The ability of IgG to potentiate Ag presentation has been demonstrated *in vitro* (9, 124-127) and *ex vivo* (124, 125), and has been implied *in vivo* (135, 161, 162). To directly visualize the effect of IgG2a/Ag complexes on the Ag-specific T cell response *in vivo*, we adoptively transferred OVA-specific DO11.10 T cells into normal BALB/c mice. As expected immunization with IgG2a/OVA complexes induced enhanced IgG anti-OVA serum titers in the recipients. The enhanced Ab response was preceded by a rapid expansion of OVA-specific $CD4^+$ cells, peaking at day 3 post immunization. A 3.2 to 9.5 fold increase in population size was observed when compared to the response the Ag alone. Similar experiments in $FcR\gamma^{-/-}$ mice showed that, like IgG2a-mediated enhancement of the Ab response, enhancement of the T cell response is dependent on activating FcγRs (II).

FcγRIIB restrains the Ab response to IgG2a/Ag complexes

$Fc\gamma RIIB^{-/-}$ mice and wildtype controls were immunized with IgG2a/Ag complexes or Ag alone and several parameters of the Ag-specific Ab response were measured. While wildtype mice showed enhanced primary responses to IgG2a/Ag complexes (10-fold), the responses were much more enhanced (250-470 fold) in $Fc\gamma RIIB^{-/-}$ mice. When boosted with Ag alone, $Fc\gamma RIIB^{-/-}$ mice, primed with IgG2a/Ag complexes, developed stronger secondary responses. The higher Ab levels in immunized $Fc\gamma RIIB^{-/-}$ mice were reflected in higher numbers of B cells producing specific IgG and an earlier onset of germinal center formation in these mice. To get an indication of whether FcγRIIB negatively influences IgG2a-mediated T cell activation, wildtype and $Fc\gamma RIIB^{-/-}$ mice were compared in their ability to induce DTH reactions. No difference between the two strains was found.

The mechanisms controlling the Ab response to IgG2a/Ag complexes

Our data suggests that activating and inhibitory Fc receptors act at different levels during an Ab response provoked by immune complexes, consisting of IgG and soluble protein Ags. During the initial steps towards an enhanced Ab response immune complexes are taken up by activating FcγRs on APCs, probably DCs, and presented to Ag-specific T cells, provoking stronger T helper cell responses. We find it likely that FcγRIIB exerts its negative effect on the Ab response to IgG/Ag complexes on the B cell level.

There is no evidence that FcγRIIB has any negative effect at the initial Ag presentation stage. We found no difference in DTH between wildtype and $Fc\gamma RIIB^{-/-}$ mice. This suggests that FcγRIIB has no effect on the IgG/FcγR-

mediated T cell priming, which has been shown to be important in DTH (135). Furthermore, a study dissecting the role of individual FcγRs on BM-DCs, showed that FcγRIIB actually enhances MHC class II-restricted Ag presentation (136). FcγRIIB has been reported to interfere with Fc-receptor mediated DC maturation (134) and thereby reduce T cell priming. However, a detailed study suggested that the size of the immune complex determines the effect of FcγRIIB on DC maturation. Small immune complexes probably do not cause enough crosslinking for FcγRIIB to interfere with DC maturation (136).

The presence of IgG/Ag complexes attenuates the B cell response via FcγRIIB-BCR co-crosslinking on Ag-specific B cells. Judging from our data in FcγRIIB$^{-/-}$ mice (earlier germinal center formation accompanied with high numbers of specific IgG producing cells), this probably occurs during the germinal center reaction. Support for this conclusion comes from *in vitro* data where BCR-FcγRIIB co-crosslinking leads to inhibition of B-cell signaling (24, 30, 163, 164), B cell apoptosis (165), and inhibition of BCR-mediated Ag uptake and presentation (34, 35). In addition, two studies revealed a correlation between reduced levels of FcγRIIB expression on germinal center B cells (as a result of FcγRIIB promoter polymorphisms) and higher Ab responses (166, 167). A delicate role of FcγRIIB within the germinal center reaction is further suggested by the observation that germinal center B cells already have reduced levels of FcγRIIB (168). FcγRIIB expression on B cells is reduced by IL4, suggesting an additional mechanism by which T cells promote B cell activation (169). The high levels of FcγRIIB expression of FDCs within germinal centers are thought to further reduce B cell inhibition by competing for the FcγRIIB ligands within IgG/Ag complexes (170).

Even though FcγRIIB exerts its negative effects directly on B cells, it should be stressed that FcγRIIB only attenuates Ab responses and does not completely inhibit them. This can be inferred from the enhanced Ab responses to IgG/Ag complexes in wildtype mice, which do express FcγRIIB (93, 94).

CD23-mediated antigen presentation of IgE/Ag complexes by B cells prime T cells in vivo (paper III)

A likely mechanism underlying IgE-mediated enhancement of the Ab response is CD23-dependent Ag uptake and presentation to T cells, resulting in better help to B cells. At the onset of this study this hypothesis was based on two observations: 1) IgE-mediated enhancement is dependent on bone marrow-derived CD23^{+} cells, most likely B cells (115) and 2) *In vitro*

studies show that B cells take up IgE/Ag complexes via CD23 and present Ag to T cells (62-65). Here, we provide additional *in vivo* support for the proposed mechanism.

IgE/Ag complexes enhance Ab and T cell responses in a CD23-dependent manner.

The OVA-specific Ab and T cell response to IgE/OVA complexes was examined using the DO11.10 adoptive transfer system. Immunization with IgE/Ag complexes, but not Ag alone, resulted in an enhanced IgG anti-OVA response, preceded by a potent and rapid $CD4^+$ T cell response. The number of Ag-specific T cells was significantly increased at day 2 post immunization and peaked at day 3 with a 6-21 fold expansion. The observed expansion in cell numbers was due to T cell activation as we demonstrate that the T cells went through several rounds of division and changed the expression of cell surface markers from a naive to an activated phenotype. Like the enhanced Ab responses observed after immunization with IgE/Ag complexes, the enhanced T cell responses were absent in the absence of CD23 but not FcεRI (III).

$CD23^+$ B cells are responsible for IgE-mediated enhancement of Ab and T cell responses.

Several cell transfer strategies confirmed that $CD23^+$ B cells are responsible for IgE-mediated enhancement. Transfer of $CD23^+$ spleen cells to $CD23^{-/-}$ mice enabled recipients to respond to IgE/Ag complexes with enhanced Ab and T cell responses. The ability to confer IgE-mediated enhancement was lost when the $CD23^+$ cell population was depleted of $CD19^+$ cells.

Mast cells have been shown to express CD19 (171) and are capable presenting antigen (172), therefore we wanted to excluded that our $CD19^+$ effector cells were mast cells. Most hematopoietic cells, including mast cells, express CD43 whereas naive B cells do not. We isolated B cells by magnetic depletion of $CD43^+$ cells and confirmed B cell dependence by transferring purified $CD4^+$ DO11.10 cells and purified $CD23^+$ or $CD23^-$ B cells ($CD19^+$ $B220^+$ IgM^+ $CD43^-$) into $CD23^{-/-}$ mice. IgE/Ag complexes induced an enhanced T cell response only in mice receiving $CD23^+$ B cells.

The mechanism controlling the antibody response to IgE/Ag complexes.

One of the characteristics of the (enhanced) Ab response after immunization with IgE immune complexes is a rapid IgG response. Six days after immunization, the number of B cells producing Ag-specific IgG and the serum levels of Ag- specific IgG reach their peak (114). A likely explanation for its efficiency can be found in the central role of the B cell in IgE-mediated enhancement.

The majority of freshly isolated splenic B cells, regardless of Ag specificity, bind IgE immune complexes via CD23 (147). Efficient targeting of the Ag to B cells by means of IgE should increase the likelihood of Ag-specific B cells getting activated.

Here we show that IgE/Ag complexes induce a rapid and potent Ag-specific $CD4^+$ T cell response *in vivo*. IgE's potency to initiate T cell responses can be illustrated by the observation that a 100-fold higher dose of OVA with LPS *s.c*. is required to obtain a comparable degree of T cell expansion (173). Both the ability of IgE to enhance Ab responses and its ability to enhance T cell responses are dependent on CD23-expressing B cells. This is a strong argument for the proposed mechanism of enhanced IgE-mediated Ag presentation and T cell activation by $CD23^+$ B cells, resulting in better T cell help to B cell.

As mentioned above, several *in vitro* studies demonstrate the ability of B cells to take up and present IgE-bound Ag via CD23 (62-65). Our data showing that B cells prime naïve T cells *in vivo* in a CD23-dependent manner is interesting, since it has been claimed that B cells do not prime naive T cells (174-177), but do activate Ag-experienced T cells (176, 177). Antigen presentation by B cells to naive T cells is thought to result in T cell tolerance (175-177). On the other hand, studies have shown that Ag-specific B cells can prime T cells specific for their Ag *in vitro* and *in vivo* (178-181).

Whether or not the Ag presenting $CD23^+$ B cells in our experimental system are Ag-specific is not known at the moment. It would be very interesting if B cells could present Ag acquired via an "unspecific" receptor such as a Fc receptor instead of the Ag-specific BCR. Alternatively, co-crosslinking of CD23 and the BCR on Ag-specific B cells, by IgE/Ag complexes, could enhance the Ag presenting capacity of the B cell via its BCR.

In conclusion, our results show that $CD23^+$ B cells play a central role in the mechanism underlying IgE-mediated Ab enhancement. They induce a potent Ag-specific $CD4^+$ T cell response and an enhanced antibody response.

Conclusions

Based on this thesis the following conclusions can be drawn concerning the mechanisms underlying antibody feedback regulation:

1. IgG-mediated suppression *in vivo* is not dependent on the presence of FcγRIIB. IgG will most likely suppress the Ab response to

particulate Ags by sterically hindering the interaction between the Ag and the BCR and by promoting Ag clearance (I).

2. The ability of FcγRIIB to negatively regulate the Ab response is most likely the result of a direct inhibition of the B cell via co-crosslinking of the BCR and FcγRIIB by IgG/Ag complexes. Instead of completely inhibiting antibody responses it attenuates the magnitude of the Ab response (I & II).
3. IgG/Ag complexes induce a potent Ag-specific $CD4^+$ T cell response *in vivo* in a FcγR-dependent manner. The enhanced T cell help to Ag-specific B cells is probably a major mechanism underlying the observed IgG-mediated enhancement of the Ab response (II).
4. IgE-bound Ag is taken up via CD23 and presented by B cells to T cells, resulting in a potent Ag-specific $CD4^+$ T cell response and enhanced Ab responses (III).

General discussion.

Long after an immune response has subdued, Ag-specific IgG is detectable in the serum. This is probably due to long-lived plasma cells (182). During a second Ag exposure, the Ag-specific IgG will bind the Ag to form immune complexes, increasing the likelihood of interaction between Ag and Fc receptor-positive cells of the immune system. Ag bound to Fc receptors on FDCs (183) and B cells (147), will increase the Ag concentration within the B cell follicles, and hereby increase the chances of Ag-specific B cells encountering their Ag. Meanwhile the Ag is also targeted to FcR-positive APCs. These APCs will take up immune complexes, receive maturation stimuli (5, 124, 125, 133), and migrate to lymphoid organs, if they do not already reside there. There, the Ag will be presented to Ag-specific T cells and induce a potent T cell response (II, III). As a result IgG will promote Ag-specific immune responses at low Ag concentrations.

IgG can have a dual effect on the Ab response. Monoclonal TNP-specific IgG administered with KLH-TNP enhances the response to KLH, whereas the same Abs administered with SRBC-TNP suppresses the response to SRBCs (92). A physiological role for suppression by means of epitope masking could lie in modulating the B cell repertoire. Binding of IgG to the epitopes which elicited their production would hide them from BCRs and thereby increase the chances of less immunogenic epitopes being recognized. This may broaden the spectrum of the Ab response. Similar mechanisms may operate on the T cell level. *In vitro* studies have shown that binding of specific IgG to model Ags, such as thyroglobulin and tetanus toxin, suppresses presentation of certain T cell epitopes while enhancing presentation of others (184, 185).

Work presented in this thesis shows that IgG-immune complexes induce potent T helper cell responses (II, III). Others have shown that besides inducing MHC class II-presentation, IgG/Ag complexes also induce antigen presentation on MHC class I and subsequent $CD8^+$ T cell responses (5, 124, 125, 134, 186, 187). The ability of IgG to activate both $CD4^+$ and $CD8^+$ T cells could play a role in anti-viral, anti-bacterial and anti-tumor responses. Recent observations within the field of bacteriology (188), virology (189) and tumor biology (125, 190) are in support of this notion.

It is easy to imagine how immune complexes can be involved in the pathogenesis of autoimmunity. In autoimmunity, IgG-mediated feedback enhancement would serve as amplification during the initial stages of disease development, stimulating the production of auto-reactive Abs initiating the outbreak of disease. Since FcγRIIB controls the magnitude of the Ab response to IgG/Ag complexes (94, 191), disruptions in its function would promote autoimmunity. Indeed FcγRIIB$^{-/-}$ mice develop both spontaneous autoimmune disease (40, 41) and more severe induced autoimmune disease (17, 23, 38, 39). Accordingly, polymorphisms within the FcγRIIB regulatory regions of autoimmune-prone mouse strains correlated with down-regulation of FcγRIIB expression on germinal center B cells and up-regulation of IgG antibody responses (166, 167). Additionally, IgG/Ag complex-mediated T cell activation has been suggested to play an important role in disease development in an animal model for multiple sclerosis (192).

Some of the physiological roles of IgG-immune complexes, described above, will probably also apply for IgE-immune complexes. However, the physiological function of IgE itself is not completely clear. A role for IgE in immunity to helminthic parasites has been suggested, although IgE is best known for its role in allergy. The serum concentration of IgE is very low (~150 ng/ml), but observations in patients with different kind of allergies suggest that IgE is mainly produced locally at the site of allergy ((193) and references therein). At such sites, IgE-mediated antigen presentation (194) and Ab enhancement could result in a positive feedback loop, strengthening the allergen-specific T cell and Ab responses. Support for a role for IgE-mediated antigen presentation in allergy comes from animal studies (195, 196). Moreover, van der Heijden et al used an autologous system, where B and T cells were derived from the same atopic dermititis patient, to show that patient serum containing IgE anti-Der p II (major house dust mite) facilitated EBV-B cell-mediated antigen presentation to T helper cells *in vitro* (65).

The identification of CD23^{+} B cells as effector cells in IgE-mediated enhancement is of particular interest as *in vitro* studies suggest that B cell-mediated T cell activation favors the induction of Th2 cells (197, 198), thought to be important in the development of allergic disease. Whether or not IgE immune complexes skew T cell responses towards a Th2-type has not been tested directly yet. The isotypes of the Ag-specific Abs, produced during IgE-mediated enhancement show that both Th1 (IgG2a) and Th2 (IgG1)-associated isotype-serum levels are elevated, although IgG1 levels were most potently enhanced (199). As discussed in the "present investigation" section, it is somewhat controversial whether B cell present Ag to naive T cells. At present we do not know if all CD23^{+} B cells present IgE/Ag or if only Ag-specific CD23^{+} B cells have this capacity. If Ag-

unspecific B cells present IgE-bound Ag in a CD23-dependent manner, it would be very interesting to know how they keep their two different functions as APC and potential Ab producing cell separated. Probably only Ag-specific B cells would get the necessary activation signals via its BCR to be able to respond to activated T helper cells and become activated themselves.

The most interesting question remains, namely why this B cell function exists. Obviously, CD23-mediated antigen presentation by B cells would be restricted to Ags provoking an IgE response. Detailed studies on IgE responses show that they may originate in the gut-associated lymphoid tissue (200). Possibly IgE plays a role in mucosal immunity. Within the context of food allergy CD23 has been shown to facilitate transport of IgE-bound Ag over intestinal epithelia (80, 81). Assuming that B cells have an intrinsic ability to provoke Th2 types of responses, IgE-mediated antigen presentation by B cells would locally amplify Th2 T cell responses and the production of IgG1 and IgE. This might be beneficial in the response to helminthic parasites.

This thesis has described some of the mechanisms underlying Ab feedback regulation. Improved understanding of Ab feedback regulation will lead to a better understanding of secondary responses, in which its most important physiological role lies. It will also give us more insight into humoral responses in newborns, in which passively transferred maternal Abs can influence antibody responses.

With the knowledge we have today, it is impossible to think of Abs only in terms of effector molecules, involved in downstream functions such as neutralizing Ag and promoting leukocyte-dependent destruction of microbes. Abs actively participate in the initial phases of immune responses by regulating their own production, both in quantity and quality, and influence $CD4^+$ and $CD8^+$ T cell responses. In addition they form an important part of immunological memory. We are only beginning to understand the impact of Abs on the immune response.

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