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A Search for the Masked Mechanism Behind IgG-Mediated Suppression of Antibody Responses

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Abstract
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Antibodies passively administered together with their specific antigen can enhance or suppress the specific antibody response. This phenomenon is known as antibody feedback regulation. Whether this modulation causes up- or downregulation of the antibody response depends both on the antibody isotype and the antigen used. IgG antibodies passively administered together with particulate antigens, e.g. erythrocytes, can completely prevent the induction of an antibody response to the antigen. The suppressive capacity of IgG has been routinely used in the clinic since the 1960's in RhD-prophylaxis to prevent hemolytic disease of the fetus and newborn. Although studied for decades, the underlying mechanism of IgG-suppression has remained elusive. The main focus of this thesis has been to elucidate the mechanism behind IgG-suppression of antibody responses *in vivo* in mouse models using intravenous immunization with specific IgG together with native or haptenated sheep red blood cells, SRBC. We show that IgG-suppression of IgM and long-term serum IgG-responses operates independently of activating FcγRI, III, IV, or the inhibitory FcγRIIB, thus confirming and extending previous findings. Moreover, we demonstrate for the first time that C1q, C3 and CR1/2 are dispensable for IgG-suppression of antibody responses. These findings strongly argue against the involvement of Fc-dependent mechanisms as the explanation for IgG-suppression. Interestingly, GC formation occurs in IgG-suppressed mice although the antibody response to surface SRBC epitopes are completely suppressed. The data suggests that these GCs develop in response to intracellular SRBC epitopes as well as to the passively administered suppressive IgG. Moreover, we demonstrate that passively administered IgG suppresses several parameters of an antibody/B cell response including antigen specific GC and non-GC B cells, extra-follicular antibody secreting cells, long-lived plasma cells and induction of immunological memory. Before the onset of the present study, two mechanisms appeared compatible with the majority of experimental findings: IgG-mediated antigen clearance and epitope masking. Herein we show that the contribution of IgG-mediated antigen clearance is negligible and that suppression of IgG-responses is strictly epitope specific. This provides compelling evidence that a very important mechanism underlying IgG-suppression is epitope masking.

Keywords: FcγR, complement, sheep erythrocytes, IgG-mediated immune suppression, rhesus prophylaxis, rhesus D antigen, germinal center

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Don't you think
if I were wrong, I'd know it?

Dr Sheldon Cooper,
The Big Bang Theory

List of Papers

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

I **Bergström, J.J.E.** and Heyman, B. (2015). IgG suppresses antibody responses in mice lacking C1q, C3, complement receptors 1 and 2 or IgG Fc-receptors. *PLoS One*, 10(11): e014384.

II **Bergström, J.J.E.** and Heyman, B. Development of germinal centers in mice immunized with IgG anti-SRBC and SRBC in spite of a completely suppressed SRBC-specific antibody response. *(manuscript)*.

III **Bergström, J.J.E.***, Xu, H.* and Heyman, B. (2017). Epitope-specific suppression of IgG responses by passively administered specific IgG: Evidence of epitope masking. *Front. Immunol.*, 8:238. * equal contribution.

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Contents

Abbreviations

ADCC	Antibody-dependent cytotoxicity
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
Bcl-6	B cell lymphoma 6
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein 1
BSA	Bovine serum albumin
C	Complement
C1q	Classical pathway activator of complement
C2	Complement factor 2
C3	Complement factor 3
C4	Complement factor 4
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CR	Complement receptor
Cr2	Gene encoding CR1/2
CSR	Class-switch recombination
CVF	Cobra venom factor
CXCR	C-X-C chemokine receptor
CXCL	C-X-C chemokine ligand
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DZ	Dark zone
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
Fc	Fragment, crystallizable
FcεR	Fc epsilon receptor
FcγR	Fc gamma receptor
FcRγ	Fc receptor common gamma chain
FcRn	Neonatal Fc receptor
FDC	Follicular dendritic cell
GC	Germinal center
HDFN	Hemolytic disease of the fetus and newborn
HOD	HEL-OVA-Duffy tandem antigen
Ig	Immunoglobulin
IL	Interleukin

IRF4	Interferon regulatory factor 4
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KLH	Keyhole limpet hemocyanine
KO	Knock-out
LZ	Light zone
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
MZ	Marginal zone
NK cell	Natural killer cell
NP	4-hydroxy-3-nitrophenyl acetyl
OVA	Ovalbumin
PALS	Periarteriolar lymphoid sheath
PAMP	Pathoge-associated molecular pattern
PE	Phycoerythrin
PFC	Hemolytic plaque-forming cell
pMHC	Peptide MHCII
RhD	Rhesus D antigen
SRBC	Sheeo red blood cell
SHM	Somatic hypermutation
TCR	T cell receptor
T_{FH}	T follicular helper cell
T_{FR}	T follicular regulatory cell
TGF-β	Transforming growth factor beta

Introduction

The immune system is a complex organization of cells and soluble compounds that has evolved to defend hosts against pathogens and foreign substances. This complex defense system is divided into two parts: the innate and adaptive immune systems. Innate immunity is conferred by physical barriers such as the skin and mucus layers together with soluble compounds including the complement system (C), anti-microbial peptides and various proteases. There is also a cellular component of innate immunity that includes leukocytes such as monocytes, macrophages, dendritic cells (DC), granulocytes, mast cells and natural killer (NK) cells. Although limited, the specificity of the innate immune system is based on the recognition of pathogen- and danger-associated molecular patterns (PAMPs and DAMPs). The innate immune system responds rapidly after invasion by pathogens and provides the first line of defense. To keep up with the "arms race" of rapidly mutating pathogens, a system with higher specificity and adaptability is needed, and this is provided by the adaptive immune system. Adaptive immunity, in contrast to innate immunity, takes a longer time to reach full capacity but provides the necessary specificity. The highly specific nature of adaptive immunity is provided by rearranged receptors expressed on T and B lymphocytes, which allow these cells to recognize a specific region or epitope of a particular antigen. Activation of these cells leads to the production of cytokines and antibodies that contribute to antigen clearance. Another important property of adaptive immunity is the generation of immunological memory during the primary response by producing certain effector cells called memory cells. These cells are more easily activated and initiate a stronger response after re-encountering the same antigen, thereby providing long-term immunity. Although they are seemingly separate, there is extensive crosstalk between the innate and adaptive immune system mediated by soluble compounds and cell-cell contacts. This communication ensures that the encountered antigen is removed rapidly and with high specificity. To ensure that the immune system reacts specifically to foreign antigens and not to self, several levels of control are needed. Moreover, antibodies regulate their own production through antibody feedback, thus providing yet another level of regulation. This thesis focuses on how passively administered antigen-specific antibodies affect the antibody response, and particularly how immunoglobulin (Ig) G antibodies can completely suppress antibody responses to erythrocytes.

Structure of the mouse spleen

The spleen, one of the main sites of blood filtration and the body's largest secondary lymphoid organ, is involved in the initiation of adaptive immune responses. It is highly vascularized and is located in the abdominal cavity, surrounded by a fibrous capsule. The primary functions of the spleen are to clear old/damaged erythrocytes and blood-borne pathogens from the circulation and to initiate adaptive immune responses against these antigens present in the bloodstream (1). Thus, the spleen is an organ of interest in studying immune responses to intravenously administered blood-borne antigens. Moreover, the structure of the mouse spleen is comparable to that of the human spleen, with the exception that the marginal zone (MZ) in the human spleen comprises two layers, whereas that of the mouse spleen has a single-layer marginal zone. This high similarity makes mice a good model for studying splenic immune responses. The vascularized organization of the spleen can be compared to a tree-like structure with branching arterial vessels, smaller arterioles and venous sinuses. In addition, the spleen is divided into two main compartments, red pulp and white pulp, which have distinct functions. In red pulp, blood is filtered by its passage through macrophage-containing cords lined with fibroblasts. Owing to the organization of the vascular network, the blood flow slows down and is forced into venous sinuses and collected in efferent veins (1). One of the main functions of $F4/80^+$ red pulp macrophages is to clear old/damaged erythrocytes by phagocytosis. The degradation of erythrocytes in phagolysosomes results in the release of iron, which is either secreted or recycled. Recycling of iron is important not only in iron homeostasis but also in limiting bacterial growth (2, 3). The removal of foreign erythrocytes is also mediated by red pulp macrophages, and this clearance is dependent on CD47-SIRPα interactions (4-6). The lack of CD47 results in rapid clearance of the erythrocytes (5). Moreover, the lack of CD47-SIRPα interactions may explain the strong immune response after immunization with sheep red blood cells (SRBC) (6). SRBC do not engage SIRPα on the $CD4^+$ DCs, thus resulting in activation of the DCs and uptake of SRBC. The DCs then present SRBC peptides to T cells, thereby initiating an immune response. In addition, DC activation and RBC uptake can also occur with CD47 KO RBCs (6).

The white pulp is the compartment where the T and B lymphocytes are located. The organization of the white pulp is dependent on specific chemokines that ensure the correct localization of various immune cells (1, 7). T cells localize primarily to the T cell zone, also known as the periarteriolar lymphoid sheath (PALS), which is proximal to the central arteries. Near the T cell zone, follicular B cells organize together with follicular dendritic cells (FDC) in dense structures called B cell follicles (1). During responses to thymus-dependent (T-dependent) antigens, germinal centers (GC) form in the center of the B cell follicles and are the prime sites for clonal expansion

and affinity maturation (8, 9). Bordering the B cell zone and distal to the T cell zone is the MZ, which contains macrophages, DCs and MZ-resident B cells. The border between the MZ and B cell follicle can be defined by the presence of $CD169^+$ (MOMA) metallophilic macrophages (1).

Mouse IgG Fc-receptors

The biological function of antibodies, i.e., binding to their specific antigen and forming an immune complex, is primarily mediated by their Fc. Through their Fc regions, antibodies can bind to various Fc receptors or activate C (10, 11). IgG primarily elicit their Fc-mediated functions by interacting with Fc-gamma receptors (FcγR) expressed on various cell types. In mice, there are five Fc receptors for IgG (Fig. 1): FcγRI, FcγRIIB, FcγRIII, FcγRIV and the neonatal Fc receptor (FcRn) (11, 12). The specificity of the FcγRs for different subclasses of IgG varies. FcγRI is a high-affinity receptor for IgG2a, a low-affinity receptor for IgG2b and a very low-affinity receptor for IgG3; FcγRIIB and FcγRIII are low-affinity receptors for IgG1, IgG2a, and IgG2b; FcγRIV is a high-affinity receptor for IgG2a and IgG2b; and FcRn binds all subclasses of IgG with high-affinity (13-15). FcγRs are widely expressed on different endothelial and hematopoietic cells and have distinct functions that are mediated by signaling events after engagement of the receptors. Signal transduction relies on motifs associated with each receptor. FcγRI, III, and IV are classified as activating FcγRs because of their associated FcRγ-chain, which contains an immunoreceptor tyrosine-based activation motif (ITAM), whereas FcγRIIB is classified as an inhibitory FcγR because of its immunoreceptor tyrosine-based inhibition motif (ITIM) (11, 12, 14). FcRn lacks a signaling motif, and its main functions are to protect IgG from proteolytic degradation, assist in the transport of IgG, and facilitate phagocytosis of bacteria by neutrophils (16, 17). Activating FcγRs have been shown to be involved in multiple processes, including DC maturation, antigen uptake and presentation on major histocompatibility complex (MHC) class I and II (18, 19). In addition, FcγRs on FDC contribute to GC responses by retaining immune complexes (20-22), and FcγRs on NK cells are involved in antibody-dependent cellular cytotoxicity (ADCC) (23, 24). Moreover, a well-established function of activating FcγRs is to induce activation and antibody-dependent phagocytosis by macrophages (14, 25, 26). In contrast, FcγRIIB on the surface of B cells inhibits B cell activation by ITIM signaling by co-crosslinking with the B cell receptor (BCR) and consequently recruiting phosphatases such as SHIP and SHP1, which interfere with BCR-mediated activation by disrupting the recruitment of kinases responsible for downstream signaling events (27-29). In knock-out mice, it has been observed that the deletion of FcγRIIB results in augmented antibody responses, a higher risk of developing autoimmune diseases and higher sensi-

tivity to anaphylaxis (30). These observations clearly demonstrate the role of FcγRIIB in negatively regulating immune responses.

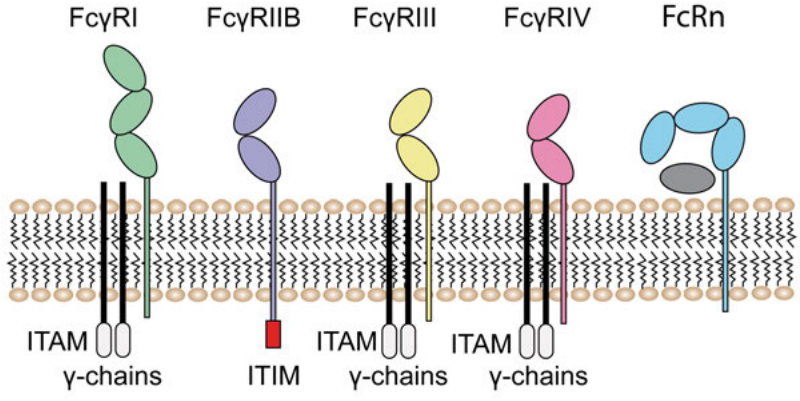

Figure 1. **Mouse IgG Fc-receptors.** There are five FcRs for IgG with varying specificities for the different IgG subclasses. FcγRI: IgG2a>>IgG2b>IgG3; FcγRIIB: IgG1=IgG2a=IgG2b; FcγRIII: IgG1=IgG2a=IgG2b; FcγRIV: IgG2a=IgG2b; and FcRn binds all IgG subclasses with high affinity. FcγRI, III and IV are classified as activating FcγRs and are associated with ITAM-containing γ-chains. FcγRIIB is an inhibitory FcγR associated with an ITIM.

Complement in regulation of antibody responses

The importance of C in adaptive immune responses has been clearly demonstrated in mice depleted of complement factor 3 (C3) through the injection of cobra venom factor (CVF), which results in a severely impaired antibody response (31). Moreover, impaired antibody responses have been observed in animals and humans lacking C1q, C2, C3, or C4, owing to hereditary defects or gene targeting (32-35). The classical, alternative and lectin pathways of complement activation all share C3 as their central factor. Interestingly, deletion of factor B, a component upstream of C3 in the alternative pathway, does not affect antibody responses (36). Because the deletion of C1q, which is involved in only the classical pathway, leads to severely impaired antibody responses (34, 37), it is likely that classical pathway activation is crucial to the generation of antibody responses.

The classical pathway is activated by IgM or IgG antibodies bound to their specific antigens, thus leading to the formation of the C3 convertase, which cleaves C3 into C3 split products. These split products are the ligands for complement receptors 1 and 2 (CR1/2). CR1/2 in mice are encoded by the same gene (Cr2) and are produced as the result of alternative splicing. CR2 is expressed primarily on B cells whereas CR1 is expressed primarily on FDC (38). Furthermore, blocking or deleting CR1/2 results in an equally

impaired antibody response, as seen in C3-deficient animals (39-41), thus suggesting that the main function of C3 in antibody responses is to supply the ligands for CR1/2. How C and especially CR1/2 influence antibody responses is not completely understood, and several mechanisms have been proposed: (I) a CR1/2-mediated increase in antigen deposition on FDC (42, 43), (II) an increase in the transport of antibody-antigen complexes by B cells expressing CR1/2 (44, 45), (III) an enhanced antigen presentation to T cells by CR1/2-expressing B cells (46), or (IV) an increase in B cell activation through co-crosslinking of CR2 and BCR, thus lowering the threshold for activation (47-49). Interestingly, antibody responses have been found to be normal in a knock-in mouse carrying a point mutation in its IgM that prevents C activation. This finding suggests that the importance of C in antibody responses cannot be explained simply by activation of the classical pathway by IgM (37).

B cell activation and fate decisions in early differentiation

B cell activation, leading to the subsequent production of antigen-specific antibodies and the induction of immunological memory, classically initiates within the follicles of secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches, and is triggered by antigen exposure. After activation, antigen-specific follicular B cells have the potential to differentiate into different types of effector cells: (I) short-lived antibody-producing plasma cells, (II) GC B cells, or (III) GC-independent memory B cells. Here, the early steps during B cell responses to T-dependent antigens are discussed, with a focus on the events that influence the differentiation fates after B cell activation. The steps during the response of B cells to T-dependent antigens are summarized in Fig. 2.

B cells and T cells are physically separated within secondary lymphoid organs on the basis of their differential expression of the C-X-C chemokine receptor type 5 (CXCR5) and the C-C chemokine receptor type 7 (CCR7). B cells express high levels of CXCR5 and are drawn to the follicular stroma of the secondary lymphoid organs by chemotaxis mediated by the chemokine CXCL13, which is produced by FDC (50-52). B cells also express low levels of CCR7, which is a receptor for CCL19 and CCL21, both produced in the T cell zone (53, 54). Resting T cells express the complementary phenotype ($CXCR5^{lo}$ $CCR7^{hi}$) and are therefore restricted to T cell zones (55, 56). After antigen exposure, the signaling initiated by the engagement of the BCR with its specific antigen causes an increase in the expression of CCR7 on the surfaces of B cells (57). Moreover, BCR-antigen engagement triggers endocytosis of the BCR-antigen complex, thus allowing for the processing and

presentation of the antigen on MHC II to cognate $CD4^+$ T cells. In parallel, $CD4^+$ T cells, receiving activating signals from antigen presenting cells (APC) (58), increase their expression of CXCR5 (55, 59). As a result of these changes in chemokine receptor expression, antigen-binding B cells and activated T cells are guided to the border between the B cell follicle and T cell zone (55, 57, 60).

At the T-B cell border, the B cells activated by antigens present antigen in the form of peptides loaded on MHC II (pMHC), thus further activating antigen-specific $CD4^+$ T cells. The cognate interaction between pMHC and the T cell receptor (TCR) induces the upregulation of the co-stimulatory molecules B7-1 (CD80), B7-2 (CD86) and CD40 on the surfaces of B cells. These molecules provide ligands for CD28 and CD40L on the interacting T cells (61-63). Together, these contacts deliver further activation and survival signals to the B cells and promote their differentiation and proliferation. After the initial activation, and with assistance from cognate T cells, the activated B cells have the potential to undergo differentiation into three types of cells through alternative differentiation pathways: (I) short-lived antibody producing plasma cells, (II) GC B cells, or (III) GC-independent memory B cells. Interestingly, the progeny of a single naïve B cell have the ability to generate all three types of effector cells, thereby suggesting that their fate is not predetermined (64-66).

The differentiation of plasma cells is triggered by an increase in the expression of the transcription factors B lymphocyte-induced maturation protein 1 (Blimp-1) and interferon regulatory factor 4 (IRF4) (67, 68). The early extra-follicular plasma cells/plasmablasts reside outside the B cell follicles in extra-follicular foci and secrete early antibodies (69). The secreted antibodies primarily are of the IgM isotype, although some class-switching to other isotypes can occur. Moreover, the secreted antibodies display low degrees of affinity maturation and therefore are usually of low to modest affinity (70). An essential regulator of GC initiation and maintenance is the transcription factor B cell lymphoma 6 (Bcl-6) (71). B cells committed to the GC fate have selectively upregulated Bcl-6 levels and rely on its expression to maintain the GC B cell phenotype (72, 73). This phenomenon has been demonstrated in Bcl-6-deficient mice, which are unable to form GCs (73, 74). The decision of whether the early plasma cell/plasmablast differentiation or GC induction occurs depends on the relative expression of Blimp-1 and Bcl-6. Blimp-1 and Bcl-6 repress each other and therefore provide a transcriptional switch dictating whether a B cell differentiates through the plasma cell or GC pathway (68, 71, 75). Moreover, IRF4 represses Bcl-6 expression (76). Plasma cell/plasmablast differentiation is thought to be the default fate of activated B cells, and this process is linked to cell division (77, 78). During early clonal expansion, there is a probability for each daughter cell to differentiate toward the extra-follicular plasma cell/plasmablast fate. The expansion of this population is linked to the number of cell divisions and is aug-

mented by cytokines produced by T cells including interleukin (IL)-2, IL-6 and IL-21, which further promote Blimp-1 expression (79, 80) Moreover, the affinity of the BCR may play a role in this fate decision. One study has shown that higher affinity B cells preferentially differentiate into extra-follicular plasma cells (81), whereas another study showed that the BCR affinity is linked to expansion and survival rather than direct differentiation of the activated B cells into the extra-follicular plasma cell fate (64) or both (66). The latter results are consistent with the proposed mechanism that differentiation and expansion of extra-follicular plasma cells occur in a probabilistic process linked to cell division, although the details surrounding this decision are not fully understood.

After antigen recognition and interaction with cognate T helper cells at the T-B cell border, some activated B cells undergo terminal differentiation into GC B cells, in a manner mediated by the expression of Bcl-6. These cells migrate back to the follicular area and begin to rapidly proliferate and initiate the GC reaction (71).

The factors influencing the fate decision between the GC B cell and GC-independent memory B cell fate are not fully understood. However, T cell interactions appear to be essential in this process, because blocking the CD40-CD40L interaction with antagonistic anti-CD40L antibodies inhibits the formation of GCs while promoting the differentiation of GC-independent memory B cells (82).

The germinal center reaction

GCs are the major sites of somatic hypermutation (SHM), class-switch recombination (CSR), and the production of long-lived antibody producing plasma cells and memory B cells (8, 9). GCs are highly dynamic structures that form within the central area of the B cell follicles of secondary lymphoid organs, and their function depends on the interactions among different cell types such as B cells, antigen-retaining FDCs and T cells (21, 83-86).

The need for T cells during GC initiation and maintenance

The assistance of cognate T cells is essential for the initiation and maintenance of GCs and for the generation of class-switched high-affinity antibodies from GC B cell clones that have undergone SHM. Several experimental findings support these conclusions. First, athymic nude mice are unable to form GCs. This deficiency can be reverted by the adoptive transfer of thymocytes before immunization, thus suggesting an essential role for T cells in GC responses (87). Furthermore, ongoing GC reactions can be disrupted by preventing CD40-CD40L interactions by the administration of blocking antibodies, thus further supporting that assistance from cognate T cells is cru-

cial for the maintenance of GCs (88). Several T cell subsets are present in the GC (89), such as Th17, NK T cells and $CD8^+$ regulatory T cells (90-92). How these subsets contribute to the GC reaction is not fully understood. More is known about the GC-residing $CD4^+$ T cell subsets expressing Bcl-6 and high levels of CXCR5, namely, the $Foxp3^-$ T follicular helper cells (T_{FH}) and $Foxp3^+$ T follicular regulatory cells (T_{FR}). T_{FH} are thought to be fundamentally important in providing crucial signals to cognate GC B cells, thereby promoting their selection and playing an essential role in driving affinity-based selection in GCs (85, 86, 93, 94). Moreover, studies using intravital microscopy have suggested that antigen peptide presentation by GC B cells to T_{FH} is the limiting step during the affinity-based selection of GC B cells (85, 86, 94). Furthermore, an altered T_{FH} population can cause aberrant GC formation (95). In contrast, T_{FR} negatively regulates the GC reaction by suppressing cytokine production by T_{FH} as well as antibody production and CSR by B cells (96-98).

Initiation of GC formation

What factors dictate which B cell clones will be given the opportunity to form GCs? Access to the GC is determined by interclonal competition and is directly linked to the affinity of the BCR, and GCs can form from clones of varying affinities (86, 99, 100). Additionally, low-affinity B cells can generate GCs only in the absence of competition from higher affinity clones binding the same antigen, thus suggesting that the affinity threshold for the differentiation of GC B cells is very low (101). One explanation for this observation is that higher affinity B cells would bind more antigen and subsequently present more pMHC to T cells. The T cells would preferentially interact with the B cells displaying the highest density of pMHC, thus providing the necessary signals for further B cell activation and GC B cell differentiation (101). In a competitive setting, this scenario would result in the low-affinity clones that are intrinsically capable of forming GCs to be outcompeted for T cell help by higher affinity clones binding the same antigen (101). Moreover, clonal diversity during GC initiation appears to be determined by antigen-related properties, because the diversity of founder cells in the GC varies depending on the antigen used, thus suggesting that there is no strict limit for the amount of founder cells in the GCs (102-104).

Germinal center polarization: dark zone and light zone

The interplay between T and B cells is just one example illustrating the intricate cellular dynamics taking place within GCs. Another fascinating and important characteristic of GCs is its anatomical polarization into a dark zone (DZ) and a light zone (LZ) after GC maturation (105). The DZ and LZ differ in their microanatomy, and their names stem from historical histologi-

cal observations. The DZ, localized proximal to the T cell zone, is characterized by its dark color, which results from the presence of a dense population of proliferating cells called centroblasts. In contrast, the LZ, localized distal to the T cell zone, close to the MZ in case of the spleen, does not have the dense cell population seen in the DZ. The LZ contains fewer cells, centrocytes, interspersed within a network of antigen-retaining FDCs (105), thus giving the LZ its characteristic light color in histology. In addition to the anatomical differences observed by histology, the DZ and LZ are functionally different (86, 106, 107). The DZ is primarily comprised of rapidly dividing GC B cells undergoing activation-induced cytidine deaminase (AID)-driven SHM, whereas the LZ contains a network of antigen-retaining FDCs and T_{FH}. On the basis of these observations, an initial model was proposed, which suggested that clonal expansion and affinity-based selection are compartmentalized within the GC (108). This model was further supported by the characterization of the two GC B cell subsets present in the DZ and LZ. DZ and LZ GC B cells are similar in terms of morphology and dynamic behavior (106, 107), but they differ in several important aspects. First, it has been observed that CXCR4 is essential for retaining a subset of GC B cells within the DZ (109). However, the CXCR4 expression alone is not sufficient to separate these GC B cell populations through flow cytometry. Moreover, *in situ* photoactivation has shown that these cells can be phenotypically separated on the basis of their relative surface expression of CXCR4, CD86 and CD83. The DZ GC B cells are $CXCR4^{hi}$ $CD86^{lo}$ $CD83^{lo}$ and LZ GC B cells are $CXCR4^{lo}$ $CD86^{hi}$ $CD83^{hi}$ (86). The higher expression of the activation markers CD86 and CD83 suggests a more activated state in the LZ GC B cells. This phenomenon was further confirmed by the observed activation of c-Myc and NF-κB together with specific genetic signatures associated with CD40 and BCR stimulation in these cells (86). These findings are compatible with the proposed model that GC B cells are activated in the LZ, and they further support the notion that affinity-based selection occurs within this compartment (108). Furthermore, DZ GC B cells are primarily in the G2/M phase of the cell cycle, whereas cells in the G2/M phase are nearly absent in the LZ. Additionally, the enrichment of G2/M cell cycle genes in the DZ compared with the LZ confirms the difference in the proliferative capacity between the GC B cell subsets in these compartments, thereby localizing proliferation and clonal expansion to the DZ compartment (86, 106). In summary, clonal expansion and affinity-based selection are indeed compartmentalized within the GCs with expansion taking place in the DZ and affinity-based selection within the LZ.

Affinity-based selection and cyclic re-entry to the DZ

After immunization, the iterative selection and expansion of rare clones of high-affinity GC B cells ensures the progression of antibody affinity over

time (8, 9). How the selection of high-affinity B cells operates within the LZ of GCs has been a topic of intense investigation. An important feature of GC B cells is that they exist in a pro-apoptotic state, owing to the suppressed expression of several anti-apoptotic factors and high expression of the death receptor Fas; furthermore, the survival of these cells is dependent on external survival signals (99, 110, 111). Initially, affinity-based selection was suggested to be driven by competition for limiting amounts of antigen retained in the form of complement opsonized immune-complexes deposited on FDCs with the competition being based on relative BCR affinity among the GC B cell clones (108, 112, 113). This model is appealing, because it can provide a simple explanation for why the selected clones are induced to survive and proliferate via BCR signaling. However, this model of selection does not consider how the GC B cells that are not positively selected for are prevented from proliferating or are induced to die by apoptosis. Moreover, the selection by BCR signaling alone seems far too simplistic in the dynamic context of the GC (106, 107). The strength of antigen recognition and epitope density have been suggested to be important for the selection process and the subsequent generation of antibody producing plasma cells (81, 83). However, competition for antigen is not necessarily the limiting step in affinity-based selection. Importantly, GCs can form and generate affinity-maturated B cells in the absence of immune-complexes deposited on FDCs if another source of antigen is available (114). Furthermore, BCR signaling is very limited in GCs, owing to high phosphatase activity (115). Using a mouse expressing the Nur77-eGFP reporter, it has recently been confirmed that GC B cells have markedly less BCR signaling than do activated B cells. However, it has also been demonstrated that BCR signaling occurs in a subpopulation of LZ GC B cells (116). This observation further supports that the competition for antigen is not the only factor driving the affinity-based selection. GC B cells displaying high levels of pMHC to T_{FH} cells are preferentially selected to proliferate and to differentiate into plasma cells, as demonstrated with intravital microscopy and targeting antigen presentation to the T_{FH} cells by MHC II in a BCR-independent manner using antigens coupled to anti-DEC205 antibodies. Moreover, the density of the pMHC displayed is directly proportional to BCR affinity, thus suggesting that higher affinity B cells endocytose more antigen and present more pMHC to a limiting amount of T_{FH}. The T_{FH} in turn screen the GC B cells and preferentially engage with the highest affinity B cells (85, 86, 94, 117). This interaction, termed linked recognition, elicits the necessary survival and selection signals to the higher affinity B cells. In a competitive setting, the lack of these signals in the lower affinity B cells induces apoptosis. Moreover, the normalization of pMHC densities by using anti-DEC205 abolishes affinity maturation, thereby suggesting that competition among the B cells is crucial (86). This mode of selection operates independently of BCR signaling and has been suggested to be the limiting step during affinity-based selection. A third mechanism that

is compatible with both T_{FH}- and BCR-driven modes of selection is antibody feedback, in which the secreted antibodies bind and potentially mask the epitopes on the antigens retained by FDCs, thus adding another layer of competition for the GC B cells (118). This competition may also explain why the clones of varying affinities are allowed to exist in the same GC, under the assumption that their BCRs are specific for different epitopes on the same antigen or that they can effectively compete with the antigen-bound antibodies.

The GC B cells receiving ample T cell help exit the LZ of the GCs as plasma cells or memory B cells. Extensive T cell help is sufficient to induce terminal plasma cell differentiation mediated by the expression of Blimp-1, which represses Bcl-6 in the GC B cells and causes the loss of GC B cell identity. High BCR affinity has also been shown to direct GC B cells into the plasma cell fate (83, 119). Under certain circumstances, plasma cell differentiation can be triggered by strong BCR signaling alone (100). However, the signals that determine memory B cell differentiation are not known, but the lower affinity GC B cells expressing Bach2 has been suggested to have higher propensities for generating memory B cells (120). A temporal model for plasma cell and memory B cell differentiation has also been proposed and has been supported by data showing that the generation of memory B cells occurs early during the response, whereas the generation of plasma cells occurs later (121). This model is compatible with the idea that the fate decisions are related to affinity, because affinity also increases over time.

The GC B cells cycle between the DZ and LZ, in a manner dependent on the amount of T cell help given to an individual B cell in the LZ. Higher affinity GC B cells, receiving extensive T cell help, are promoted to re-enter the DZ in S-phase to initiate another round of SHM, and this is followed by selection in the LZ. The number of cell cycles that an individual cell goes through is proportional to its affinity; i.e., higher affinity B cells go through more cell cycles and spend more time in the DZ than do their lower affinity competitors. Therefore, the amount of stimuli received in the LZ sets the division timer (85, 86, 102, 122), thus ensuring that the affinity increases in an iterative fashion and that higher affinity clones dominate the GC response over time. To avoid the risk that GC B cells carrying the pMHC displayed during one round of selection may be given an “unfair” advantage, in the following round of selection, the pMHC is rapidly turned over in the DZ GC B cells through ubiquitin-mediated degradation (123).

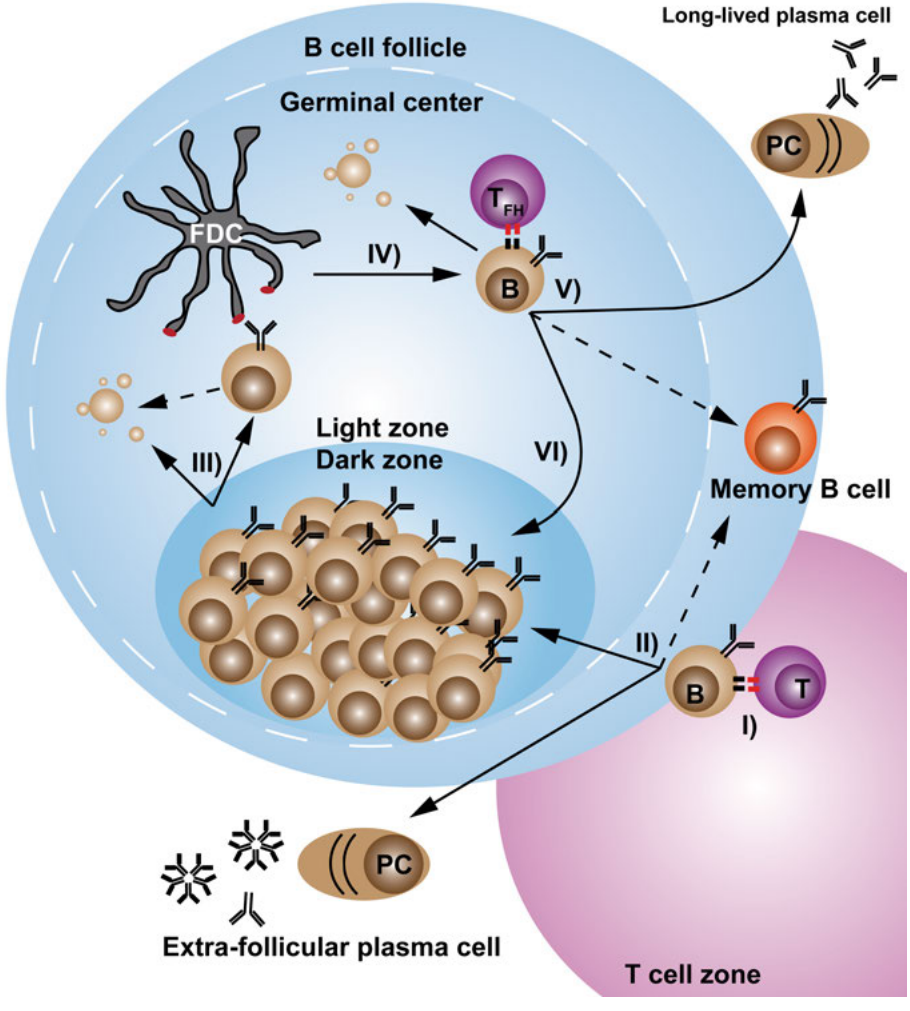

Figure 2. **B cell response to T-dependent antigens.** Dashed arrows indicate steps where the details are not fully understood. I) Activated B cells migrate to the T-B cell border and present pMHC to antigen primed $CD4^+$ T cells. II) After receiving cognate T cell help B cells can differentiate into extra-follicular plasma cells or GC-independent memory B cells. Alternatively, B cells form GCs mediated by Bcl-6. III) GCs are comprised of a DZ and a LZ. After the initial round of proliferation and SHM GC B cells move from the DZ to the LZ. In the LZ, GC B cells interact with antigen retained by FDCs. IV) GC B cells, in the LZ, will endocytose the antigen and present pMHC to a limited number of T_{FH}. The amount of pMHC presented is proportional to BCR affinity. Thus, higher affinity GC B cells will present more pMHC than lower affinity competitors. GC B cells compete for T cell help and T_{FH} preferentially engage in contact with higher affinity GC B cells mediating selection and survival of these cells. V) Selected GC B cells can exit the GC as memory B cells or long-lived plasma cells. VI) Alternatively, GC B cells re-enter the DZ for another round of SHM and selection. The amount of cell-cycles a GC B cell goes through in the DZ is determined by signals received in the LZ. To prevent carry over of pMHC between two rounds of selection, pMHC is rapidly turned over in the DZ.

Antibody feedback regulation

B cells, as part of the adaptive immune system, assist in the removal of foreign substances and infections by producing antigen-specific antibodies. Classically, the binding of antibodies to their specific antigens elicits effector functions that subsequently eliminate the antigen. Interestingly, antibodies passively administered together with their specific antigen can modulate the specific antibody response, in a phenomenon known as antibody feedback regulation (124-127). These effects occur without adjuvants, and all immunizations are done in physiological salt solutions. Emil von Behring, who received the first Nobel Prize in physiology and medicine in 1901, first observed that specific antiserum regulates antibody responses (128). Through gradient-centrifugation to size separate an antiserum, it was later shown that different fractions of the antiserum have different effects on the immune response to SRBC. The passive administration of the 19S (IgM) fraction of an anti-SRBC-specific antiserum together with SRBC causes the enhancement of the SRBC-specific response, whereas the 7S (IgG) fraction causes almost complete suppression (129). Whether the antibodies cause up- or down-regulation of the antibody response thus depends on the antibody isotype used. Moreover, the type of antigen involved also determines whether the passively administered specific antibody enhances or suppresses the antibody response. Antigen-specific IgM antibodies cause a 10- to 1000-fold enhancement of the specific antibody response to large antigens such as SRBC, keyhole limpet hemocyanin (KLH) and malaria parasites (130-133). IgG and IgE antibodies cause the enhancement of the specific antibody response when passively administered together with a small soluble protein antigen, e.g., ovalbumin (OVA) or bovine serum albumin (BSA) (134-139). IgG perform dual functions in enhancing the responses to soluble protein antigens while suppressing the responses to particulate antigens such as erythrocytes (129, 140-143). In the following sections, the enhancing and suppressing arms of the antibody feedback regulation will be discussed, with a focus on the underlying mechanisms *in vivo*. Data obtained from *in vitro* studies will not be considered because they are often discrepant in comparison with data generated *in vivo*.

Enhancement of antibody responses by specific IgM, IgE, and IgG

Enhancement by specific IgM

It was clearly demonstrated in the 1960s that SRBC-specific IgM antibodies (in the 19S fraction of the anti-serum) can enhance the SRBC-specific hemolytic plaque-forming cell (PFC) response, which measures single cells producing SRBC-specific C-activating IgM (129). IgM enhances primary anti-

body responses as well as the induction of immunological memory. Moreover, the enhancement by specific IgM primarily functions when it is passively administered before or simultaneously with immunization with large particulate antigens such as SRBC and malaria parasites or large protein antigens such as KLH (130, 133). The enhancement by specific IgM is C dependent, as demonstrated by the following experimental findings: (I) the enhancement is abolished if the specific IgM is unable to activate C (131, 133), (II) monomeric IgM, which does not activate C, is unable to enhance antibody responses (144, 145), (III) IgM cannot enhance the antibody responses in mice lacking C3 or C1q (131, 146), and (IV) IgM does not enhance antibody responses in mice lacking CR1/2 (132, 147). Together, these results suggest that specific IgM enhances antibody responses mediated by interactions of the IgM immune complex with C and its receptors. Additionally, the requirement for large antigens also suggests that IgM must bind with all arms of the pentameric molecule to induce the conformational change necessary for binding to the activator of the classical pathway C1q. Although IgM enhances antibody responses to T-dependent antigens, it does not appear to affect the T helper cells. Immunization with specific IgM together with SRBC-OVA has been found not to enhance the OVA-specific $CD4^+$ T cell response in an adoptive transfer system using OVA-specific $CD4^+$ T cells from DO11.10 transgenic mice (133). Thus, IgM enhances antibody responses by C-dependent mechanisms without enhancing the $CD4^+$ T cell response. The current view of IgM-mediated enhancement is that IgM-antigen complexes opsonized with C-factors bind to $CR1/2^+$ MZ B cells, which transport the IgM-antigen complexes to $CR1/2^+$ FDCs.

Enhancement by specific IgE

The administration of antigen-specific IgE antibodies together with small soluble protein antigens such as OVA and bovine serum albumin (BSA) enhances the specific antibody response, GC responses, and specific $CD4^+$ T cell responses (137, 148-151). In addition to the enhancement of the primary antibody response, the specific IgE also enhances recall responses (149). There are two Fc receptors for IgE, the high-affinity FcεRI and the low-affinity receptor FcεRII (CD23). FcεRI is primarily expressed on basophils and mast cells, and cross-linking of this receptor by IgE-specific antigens causes degranulation and release of bioactive mediators and proteases (152). CD23 is expressed on the cell surface as a membrane-bound trimer, and it is different from other FcRs in that it belongs to the type-II C-type lectin family and not to the super-Ig family (153). The enhancing capacity of the specific IgE antibodies is lost in mice lacking CD23 (CD23 KO) (137, 151, 154) or by the blockage of CD23 by anti-CD23 antibodies (150), thus suggesting an essential role for CD23 in the IgE-mediated enhancement of immune responses. Studies using bone marrow chimeric mice with CD23 KO and wild-type mice have shown that $CD23^+$ B cells play the main role in eliciting

immune responses and that the expression of CD23 on FDCs is dispensable (154). Moreover, IgE immune complexes have been detected on B cells in the blood 5 min after immunization and are more efficiently transported to splenic B cell follicles where they co-localize with follicular B cells (148). An interesting unresolved question is whether the $CD23^+$ B cells merely act as antigen transporters or whether they also present antigens to T cells as seen *in vitro* (155, 156), thereby explaining the enhanced T cell response. The enhancement of $CD4^+$ T cell responses has been shown to require antigen presentation by $CD11c^+$ cells, as determined by using CD11c-DTR mice and selectively depleting their $CD11c^+$ cells with diphtheria toxin administration (157). In addition, antigen presentation by B cells requires the expression of the same MHC as the responding T cells; however, the transfer of MHC-incompatible $CD23^+$ B cells also rescues the IgE-mediated enhancement of $CD4^+$ T cell proliferation in CD23 KO mice (157). These observations suggest that B cells act primarily as antigen transporters, whereas $CD11c^+$ cells are the main APCs in the IgE-mediated enhancement. In addition, a recent study has shown that the $CD8\alpha^-$ conventional DCs are the main subpopulation of $CD11c^+$ cells causing the enhanced $CD4^+$ T cell response (158). In summary, the enhancement of the immune response by antigen-specific IgE requires the expression of CD23 on B cells as well as the presentation of the antigen by $CD11c^+$ $CD8\alpha^-$ conventional DCs.

Enhancement by specific IgG

Similarly to antigen-specific IgE antibodies, specific IgG enhance antibody and $CD4^+$ T cell responses to protein antigens such as OVA, BSA, and KLH (138, 143, 159, 160). Mouse IgG1, IgG2a, IgG2b, and IgG3 all have this capacity, although IgG3 operates differently from the other subclasses. IgG3 is an interesting subclass because: (I) it is a minor subclass in responses to T-dependent antigens but is the dominant subclass in responses to type 2 T-independent antigens (161, 162); (II) it can self-aggregate through Fc-Fc interactions (163, 164); and (III) it binds poorly to FcγRs (15, 165). Because IgG3 can activate the classical C pathway (166), probably through its ability to form complexes with neighboring IgG3 molecules by Fc-Fc interactions, IgG3-mediated enhancement probably operates similarly that by specific IgM antibodies. Indeed, a central role for C in the IgG3-mediated enhancement has been demonstrated through C3-depletion and in mice lacking CR1/2 (Cr2 KO), which severely impairs the immune enhancement by specific IgG3 (160). Moreover, this effect is intact in mice lacking FcγRs (167). A study of bone marrow chimeric mice generated from Cr2 KO and wild-type mice has recently shown that the optimal enhancement by IgG3 requires CR1/2 on both B cells and FDC, although the enhancement of the antibody response is also observed when CR1/2 is expressed on only one of the cell types (139). IgG3 immune complexes are more efficiently transported to the spleen and deposited on FDCs than the antigens alone (139). Dislocation of

the MZ B cells from the MZ by the administration of FTY720 severely decreases the level of IgG3 immune complexes bound by these B cells and the amount of antigen localizing to the splenic B cell follicles (139). These observations suggest that transport of the IgG3-antigen is performed by the CR1/2 expressing marginal zone B cells rather than by circulating B cells, as shown in IgE-mediated enhancement.

In summary, IgG3-mediated enhancement is C dependent, and the underlying mechanism involves the increased transport of IgG3 immune complexes to splenic B cell follicles by CR1/2-expressing marginal zone B cells. How the delivery of the IgG3 immune complexes from marginal zone B cells to FDCs occurs remains to be determined.

In contrast to IgG3, the enhancement by IgG1, IgG2a, and IgG2b are most probably caused by the binding of the immune complexes to activating FcγRs, because the enhancing effect is lost in KO mice lacking FcγRI, FcγRIII, and FcγRIV, owing to the lack of the common FcR γ-chain (135). In addition, the enhancement is intact in mice lacking CR1/2 (147), thus suggesting that the enhancement by IgG1, IgG2a, and IgG2b do not act through C-activation but instead acts through the binding of these IgGs to activating FcγRs. Similarly to the IgE-mediated enhancement, the enhanced response of antigen-specific $CD4^+$ T cell by IgG2a (138) requires the presence of $CD11c^+$ cells (168); however, the subpopulation of $CD11c^+$ cells that is responsible for the enhanced T cell response remains to be determined. Together, these observations suggest that the enhancement by specific IgG1, IgG2a, and IgG2b can be explained by the increased delivery of antigens in the form of immune complexes to $CD11c^+$ APCs via their binding to activating FcγRs, which in turn increases the antigen-presentation to $CD4^+$ T cells.

Suppression of antibody responses by specific IgG

Perhaps the best-known example of antibody feedback regulation is the ability of specific IgG to completely suppress antibody responses to particulate antigens such as erythrocytes. The suppression by antigen-specific polyclonal IgG is very potent, because minute amounts of IgG anti-SRBC antibodies administered before immunization with SRBC suppresses >99% of the primary IgM anti-SRBC response *in vivo,* as measured by PFC assays. Most studies of IgG suppression have been conducted using haptenated or native SRBC as the antigens (129, 141, 169-173). Although interesting in itself, IgG suppression has an important clinical application, because it has been routinely used since the 1960s to prevent hemolytic disease of the fetus and newborn (HDFN) by Rhesus prophylaxis (174, 175).

HDFN is a disease primarily caused by the incompatibility of Rhesus D antigen (RhD) between the mother and fetus (176). RhD is a membrane-associated protein encoded on chromosome 1 and is expressed on human erythrocytes. RhD displays a dominant mode of inheritance (177). In the

Caucasian population, approximately 16% of people are estimated to be RhD^- (178). RhD^- mothers carrying RhD^+ fetuses can be immunized by the RhD^+ fetal erythrocytes entering the maternal circulation via transplacental hemorrhage during delivery, abortion, or physical trauma. Immunization by fetal RhD^+ erythrocytes during the first pregnancy produces maternal IgG anti-RhD. During the second pregnancy with an RhD^+ fetus, FcRn can transport maternal IgG anti-RhD over the placenta, where it binds to fetal RhD^+ erythrocytes and causes subsequent hemolysis and HDFN (176). Before the implementation of Rhesus prophylaxis, HDFN was a substantial problem causing 150 deaths per 100 000 births (179). During the 1960s, studies in New York and Liverpool on male RhD^- subjects demonstrated that the passive administration of anti-RhD (IgG) antibodies prevents immunization with RhD^+ erythrocytes (180). After successful clinical trials, Rhesus prophylaxis was quickly implemented in routine use to prevent RhD immunization of the RhD^- mothers (181). Since its implementation, RhD prophylaxis has decreased the rate of alloimmunization to RhD by 90% and dramatically decreased the incidence of HDFN, which is now a rare disease (176). The prophylactic treatment consists of polyclonal IgG anti-RhD generated from the immunization of RhD^- male volunteers with RhD^+ erythrocytes. The downside of this approach to antibody production is that it requires human subjects to volunteer, thus limiting the supply. Additionally, there is a safety issue related to the possible risk of infection with large serum pools. Therefore, it would be beneficial to replace polyclonal IgG anti-RhD with monoclonal IgG anti-RhD. Several monoclonal IgG anti-RhD have been undergoing clinical trials, but with limited suppressive capacity, and the attempts to replace polyclonal IgG anti-RhD have been unsuccessful to date (174, 182). In addition, single monoclonal IgG antibodies have been demonstrated to be less efficient suppressors than polyclonal IgG antibodies in mouse models using SRBC as antigens (170). Interestingly, a recent study in mice has demonstrated that the same level of suppression can be achieved with a mixture of monoclonal anti-RBC antibodies, each binding to different epitopes on the same RBC, as with polyclonal anti-RBC (183). Future clinical trials with combinations of different monoclonal IgG anti-RhD are needed to determine whether this finding also applies to the humans.

IgG suppression has been studied for decades, but the underlying mechanism remains enigmatic. It is of considerable theoretical interest to understand how small amounts of IgG antibodies can completely suppress antibody responses to their specific antigens. Moreover, understanding of the underlying mechanism may contribute to the development of more efficient monoclonal anti-RhD antibodies for use in Rhesus prophylaxis.

Hypotheses to explain IgG-mediated suppression

The experimental evidence supporting the various proposed mechanisms of IgG suppression is presented below, and a more detailed discussion is presented in the general discussion (page 40). The following hypotheses have been proposed to explain how the IgG suppression of antibody responses operates:

I) Central inhibition of B cells by BCR-FcγRIIB co-crosslinking. A popular hypothesis to explain IgG suppression is based on the ability of FcγRIIB to inhibit B cell responses. FcγRIIB contains an ITIM in its cytoplasmic tail, and co-crosslinking of the BCR and FcγRIIB negatively regulates ITAM-mediated signaling via the BCR, thereby inhibiting B cell activation (184-186) (187). Therefore, IgG-antigen complexes may co-crosslink these two receptors and the lack of erythrocyte-specific antibody responses in IgG suppression may be due to the central inhibition of B cell activation by FcγRIIB.

II) FcγR- or C-mediated phagocytosis. IgG bound to erythrocytes may target the IgG-erythrocyte complexes for rapid elimination by phagocytosis mediated by the engagement of activating FcγRs expressed on phagocytes. In addition, C-opsonized IgG-erythrocyte complexes may be rapidly phagocytosed via interactions with CR3, thereby increasing antigen clearance and preventing the specific B cells from encountering their antigen.

III) C-mediated lysis. IgG might also trigger C-activation resulting in the lysis of the IgG-erythrocyte complexes. Lysed erythrocytes may be less immunogenic than intact erythrocytes, thus resulting in a lack of B cell activation.

IV) Epitope masking. IgG binding to the antigen may prevent B cells from gaining access to the antigen by steric hindrance, thereby causing unresponsiveness via epitope masking.

Suppression by F(ab')2 fragments

If IgG suppression could be explained by the first three mechanisms, IgG would act via its Fc region, whereas the fourth mechanism, epitope masking, would be Fc independent. Over the years, Fc dependence of IgG suppression has been an area of intense investigation and debate, and it is one of the key questions whose answer would unmask the elusive mechanism underlying IgG suppression. Before transgenic or KO mice were available, the Fc dependence of IgG suppression was studied by using $F(ab')_2$ fragments of IgG as suppressors. One of the initial ambiguities surrounding the importance of the Fc part of IgG arose from studies on the suppressive ability of these

fragments. $F(ab')_2$ fragments lack the Fc region and are generated by proteolytic cleavage of intact IgG by the gastric enzyme pepsin. Thus, $F(ab')_2$ cannot bind to FcγRs or activate C, thereby making it an ideal system to study the involvement of these functions in IgG suppression. Inducing suppression with $F(ab')_2$ has yielded conflicting results. Some reports have demonstrated $F(ab')_2$ as poor suppressors (159, 169, 187-189). These observations have been used as evidence for the involvement of Fc functions. Other reports have demonstrated that $F(ab')_2$ suppresses antibody responses equally well as intact IgG, thereby suggesting that IgG suppression operates via Fc-independent mechanisms (141, 190-192).

Epitope-specific or non-epitope specific suppression

Another approach to differentiate between Fc-dependent and Fc-independent mechanisms underlying suppression has been to determine whether the suppression is epitope specific or non-epitope specific. This approach, however, has also led to conflicting data, because both types of specificity have been observed. IgG sometimes suppresses antibody responses only to the epitopes to which it binds (epitope-specific suppression) (193, 194) and sometimes suppresses antibody responses both to the epitopes to which it binds and to other epitopes on the same antigen particle (non-epitope-specific suppression) (143, 159, 169, 170, 195-198). The observation of non-epitope-specific suppression has been thought to be due to Fc-dependent mechanism(s) and to exclude epitope masking, because in order for epitope masking to function, the majority of the epitopes would have to be masked by the "suppressive" and specific IgG. Therefore, the suppression of the response to the entire SRBC particle by the binding of IgG only to certain epitopes has been thought to require the Fc part and most likely to involve the inhibition by FcγRIIB or FcγR-mediated phagocytosis. In contrast, the epitope-specific suppression would suggest that the suppression can operate independently of the Fc part and therefore favors epitope masking.

Suppression and complement

Reports on the involvement of C in IgG suppression are scarce. However, it has been demonstrated that a monoclonal IgG1, unable to activate C, is an efficient suppressor of responses against SRBC (199), thereby suggesting that C activation by the classical pathway is an unlikely mechanism for IgG suppression.

Suppression in FcγR-deficient mice

Because of the confusion regarding Fc dependence caused by the discrepant results from studies with F(ab')2 fragments and of epitope specificity of suppression, Heyman and co-workers have used a new approach and tested IgG suppression in various FcγR KO mice (141). The results clearly show that the IgG suppression of anti-SRBC IgM and early IgG responses function

equally well in mice lacking the activating FcγRI, III and IV (FcRγ KO), the inhibitory FcγRIIB (FcγRIIB KO), FcγRI, IIB, III and IV (FcRγ x FcγRIIB double KO), or FcRn (β2-microglobulin KO). In addition, IgE antibodies suppress responses against SRBC to the same extent as IgG, and $F(ab')_2$ fragments are efficient suppressors of IgM anti-SRBC responses. The FcγR-independent suppression and the capacity of $F(ab')_2$ fragments to suppress have also recently been confirmed in a mouse model using allogeneic RBCs expressing the HEL-OVA-Duffy (HOD) tandem antigen (190). In addition, IgG has been shown to suppress antibody responses independently of FcγRIIB when administered several days after the antigen (140).

Suppression and clearance

Interestingly, passively administered specific IgG have been demonstrated to decrease the amounts of SRBC in the blood and spleen (198), thus suggesting the occurrence of IgG-mediated clearance. Whether this clearance contributes to the suppression of antibody responses has not been resolved. The reduction in SRBC localized to the spleen may also have profound effects on other parameters of the immune response to SRBC, including GC responses. In addition, the increased clearance of RhD^+ erythrocytes by IgG anti-RhD in Rhesus prophylaxis has been considered an important characteristic for the efficacy of the treatment, although no correlation between clearance and suppression has been observed in clinical trials of monoclonal IgG anti-RhD (174, 200). In mice, IgG suppression can occur independently of IgG-induced clearance (142).

Suppression and induction of immunological memory

Although the effect of passively administered specific IgG on primary antibody responses is well established, whether IgG also suppresses the induction of immunological memory has been a matter of discussion. Some reports have claimed that priming with specific IgG together with its specific antigen suppresses the induction of immunological memory to a similar extent as the primary response (141, 189, 201). In contrast, other studies have demonstrated that IgG has no effect on memory induction (202), or that the memory is suppressed but to a smaller extent than the primary response (203).

Present investigation

Rationale and aims

Most knowledge of the suppression of antibody responses by passively administered IgG has been gathered from experiments analyzing the early primary IgM response with PFC assays during the first week after immunization (129, 140, 141, 169, 170, 194). Much less is known about the suppression of long-term IgG anti-SRBC responses (198, 203, 204). Moreover, it is not possible to assess the suppression of B cell subsets such as extrafollicular B cells, GC B cells and non-GC B cells by simply examining the PFC or serum antibody response. Therefore, more studies are needed to address whether IgG suppresses other parameters of the immune response against SRBC.

The general aim of the studies presented in this thesis was to elucidate the mechanism underlying the IgG-mediated suppression of antibody responses to erythrocytes *in vivo*.

Paper I

By using several knock-out mouse strains, we sought to investigate whether C and FcγRs are involved in the suppression of IgM as well as long-term serum IgG responses.

Paper II

Here, we aimed to determine whether IgG is capable of suppressing germinal center responses in the spleen.

Paper III

Here, we sought to determine the contribution of antigen clearance and epitope masking in IgG suppression and whether IgG can suppress antigen-specific GC B cells, extra-follicular antibody secreting cells, long-lived plasma cells or the induction of immunological memory.

Experimental setup

Mouse strains

BALB/c: wild-type mice.
C57BL/6: wild-type mice.
Cr2 KO: lacking CR1/2 on all cell types.
C1qA KO: deficient in C1q.
C3 KO: deficient in C3.
FcγRIIB KO: lacking FcγRIIB on all cell types.
FcRγ KO: lacking FcγRI, III and IV, and FcεRI on all cell types owing to the deletion of the common FcRγ-chain.

Immunization

All immunizations were done via lateral tail vein injections with 200 µl PBS. IgG was administered 30 min before administration of SRBC or NP-SRBC. The passively administered IgG was generated in mice with a different IgG-allotype from that of the recipient mice, thus allowing for the detection of only the endogenously produced IgG.

Quantification of antibody responses

Specific serum antibody responses were assessed using enzyme-linked immunosorbent assay (ELISA) analysis. IgG allotype-specific detection antibodies were used to distinguish between the passively administered and endogenously produced IgG. The number of antibody-secreting cells and plasma cells producing specific IgM and IgG antibodies were analyzed by a direct PFC assay and enzyme-linked immunospot assay (ELISPOT).

Quantification of NP-specific B cells in the spleen

To detect antigen-specific B cells in IgG suppression, C57BL/6 mice were immunized with 4-hydroxy-3-nitrophenyl acetyl (NP)-SRBC ± IgG. The response to NP in C57BL/6 is genetically restricted, and the responding B cells primarily express BCRs with a λ_1-light chain together with a VH186.2 rearranged heavy chain (205). These characteristics enabled us to reliably detect the NP-specific B cells in the spleen through flow cytometry by detecting the $B220^+$ λ_1^+ B cells binding NP-Phycoerythrin (PE). The NP-specific extra-follicular B cells and GC B cells in the spleen binding NP-PE were quantified through confocal laser scanning microscopy.

Antigen localization in the spleen

To assess the effects of passively administered specific IgG on antigen localization to the spleen, mice were immunized with NP-SRBC and the spleens were harvested after 10 min. The samples were processed and stained with a biotinylated polyclonal IgG anti-NP produced in house followed by Streptavidin-PE detection and analyzed with confocal laser scanning microscopy.

Analysis of the splenic GC response

To analyze GC responses in IgG suppression, the spleens were collected at several time points after immunization, and analysis of the splenic GC B cells, T_{FH} and T_{FR} as well as the assessment of DZ/LZ polarization were performed through flow cytometry and/or confocal laser scanning microscopy.

Results

Here, the results of papers I-III will be presented. The discussion of the presented findings in relation to the studied mechanisms of IgG suppression is in the general discussion section (page 40).

Paper I

IgG suppresses antibody responses in mice lacking C1q, C3, complement receptors 1 and 2, or IgG Fc-receptors

Efficient suppression of primary antibody responses in C1q, C3 or CR1/2 deficient mice

With the exception of one study demonstrating that monoclonal IgG1 antibodies are equally efficient in suppressing IgM anti-SRBC responses regardless of whether they were able to activate C (199), studies of the role of C in IgG suppression have been scarce. To further study this role, wild-type control, C1q KO, C3 KO, and Cr2 KO mice were immunized with $5x10^7$ SRBC ± 50 µg IgG anti-SRBC or with 50 µg IgG anti-SRBC alone. Spleens were harvested five days after immunization, and the number of single cells producing IgM anti-SRBC antibodies was analyzed with PFC assays (Fig. 1-2, **paper I**). IgG suppressed >98% of the IgM response in the C1q KO, C3 KO, and Cr2 KO mice as well as the wild-type controls (Fig. 1-2, **paper I**). In addition, blood was collected every two weeks, and the serum IgG-response was analyzed with an allotype-specific ELISA (S1 fig., **paper I**). As expected, IgG suppressed the IgG anti-SRBC response in the wild-type controls (S1 fig., **paper I**). The suppression of IgG anti-SRBC responses in the C1q KO, C3 KO and Cr2 KO mice was difficult to assess, because these mice generally have a very poor antibody response to antigen alone (34, 35, 37, 41). By increasing the serum concentration and prolonging the substrate incubation time in the ELISA, a measurable IgG anti-SRBC response was observed in the C1q KO and C3 KO mice, and this response was efficiently suppressed by the passively administered IgG (S1 fig., **paper I**). In spite of the changes in the ELISA conditions, no IgG anti-SRBC response was detected in the Cr2 KO mice, and therefore no conclusion could be drawn about the suppression of the IgG anti-SRBC response in these animals (S1 fig., **paper I**). Thus, passively administered IgG anti-SRBC suppressed primary antibody responses without the involvement of C1q, C3, or CR1/2. These results demonstrated that C activation by IgG is not required for its ability to suppress antibody responses to SRBC.

Suppression of primary antibody responses is not dependent on the involvement of inhibitory- or activating FcγRs

IgG has been demonstrated to suppress IgM anti-SRBC and early IgG anti-SRBC responses in mice deficient in FcγRI, III, IV, FcγRIIB and FcRn (141). Whether FcγRs are involved in the suppression of long-term serum IgG anti-SRBC responses has not been investigated. To assess this question, mice lacking FcγRI, III and IV (FcRγ KO), mice lacking FcγRIIB and wild-type controls were immunized with $5x10^6$ SRBC ± 10 µg IgG anti-SRBC or with 10 µg IgG anti-SRBC alone. The IgM anti-SRBC responses were analyzed with PFC assays (Fig. 3A,C, **paper I**), and the IgG anti-SRBC were analyzed with ELISA (Fig. 3B,D, **paper I**). IgG suppressed both the IgM anti-SRBC and the long-term serum IgG anti-SRBC response in the FcRγ KO and FcγRIIB KO mice and the wild-type controls (Fig. 3, **paper I**), thereby confirming and extending previous data (141). The IgM-response to the antigen alone in the FcγRIIB KO mice was greater than that of the wild-type mice, thus confirming the negative regulatory effect of FcγRIIB on antibody responses (Fig. 3A, **paper I**) (30). Thus, the efficient suppression of IgM and long-term IgG anti-SRBC responses occurs in the absence of activating FcγRI, III, IV, or inhibitory FcγRIIB.

In conclusion, these data clearly demonstrated that IgG suppression of antibody responses operates without the involvement of FcγRs or C and strongly disfavor an Fc-dependent mechanism underlying IgG suppression.

Paper II

Development of germinal centers in mice immunized with IgG anti-SRBC and SRBC in spite of a completely suppressed SRBC-specific antibody response

Passively administered IgG anti-SRBC antibodies decrease the amounts of SRBC in the spleens of BALB/c mice (198), but whether this decrease has any implication for the immune response to SRBC is unknown. One possibility is that IgG may limit the formation of GCs, which has a strong effect on the antibody response. Here, the ability of IgG to suppress GC responses to SRBC was explored. BALB/c mice were immunized with $5x10^6$ SRBC ± 10 µg allogeneic IgG anti-SRBC or with 10 µg IgG anti-SRBC alone. The splenic GC response was followed for 14 days by using flow cytometry and confocal laser scanning microscopy (Fig. 1, **paper II**). An equally potent GC response was observed in mice immunized with IgG anti-SRBC + SRBC and with SRBC alone except 6 days after immunization, when the IgG-suppressed mice had a markedly lower GC response than the mice immunized with SRBC alone (Fig, 1, **paper II**). Importantly, immunization with IgG alone did not induce GC formation (Fig. 1, **paper II**). To ensure that the presence of GCs in the IgG-suppressed mice was not simply caused by a

reaction against the allogeneic IgG used for suppression, the GC responses in mice immunized with either allogeneic or syngeneic IgG anti-SRBC together with SRBC or with SRBC alone were compared (Fig. 2, **paper II**). Again, the formation of GCs occurred in the IgG-suppressed mice, regardless of the Ig-allotype of the suppressive IgG (Fig. 2A-L, **paper II**). The antibody response to SRBC was followed in parallel for three weeks in groups of mice immunized at the same time, and the serum IgG anti-SRBC response was analyzed by ELISA. As expected, the administration of specific IgG efficiently suppressed the IgG anti-SRBC response (Fig. 2M, **paper II**).

The ability of the GCs to polarize into a DZ or LZ GC-compartment was analyzed 6 and 10 days after the immunization, through flow cytometry of the spleens from the same mice in which GC responses were analyzed (Fig. 2, **paper II**). The IgG-suppressed mice had a near-normal DZ/LZ polarization with a slight shift in the distribution of DZ and LZ GC B cells (Fig. 3, **paper II**).

T_{FH} and T_{FR} have been shown to regulate GC responses. T_{FH} stimulate antibody responses by providing limiting help to cognate GC B cells, whereas T_{FR} down-regulates GC responses by suppressing functions of T_{FH} as well as antibody production and CSR in B cells. These findings prompt the question of whether the seemingly silent GCs in the IgG-suppressed mice are due to altered T_{FH} and T_{FR}. Therefore, we sought to determine whether the proportions of T_{FH} and T_{FR} were affected in the IgG-suppressed mice 10 days after immunization, by using flow cytometry on the spleens from the mice described in Fig. 2-3, **paper II**. No difference in the proportions of $CD4^+$ T cells or T_{FH} and T_{FR} in the spleens of the IgG-suppressed and control mice were observed (Fig. 4, **paper II**). Thus, the formation of GCs occurred in the IgG-suppressed mice, although the primary antibody responses were severely suppressed, and this effect was observed regardless of the Ig-allotype of the passively administered IgG. Moreover, the GCs displayed a near-normal DZ/LZ polarization with normal proportions of T_{FH} and T_{FR}.

Although the mice developed GCs, because the antibody response against surface determinants of the SRBC, the parameter measured by the ELISA, was suppressed by IgG, other possibilities were investigated (Fig. 5, **paper II**). The IgG-suppressed mice and mice immunized with SRBC alone generated similar IgG responses toward intra-cellular SRBC determinants (Fig. 5B, **paper II**) as well as to the allogeneic suppressive IgG (Fig. 5C, **paper II**). The reactivity toward the syngeneic IgG could not be determined, owing to technical limitations. Importantly, the mice immunized with IgG alone did not mount an anti-IgG response, thus suggesting that the IgG needed to be complexed with SRBC to be immunogenic (Fig. 5C, **paper II**).

In conclusion, passively administered IgG anti-SRBC allow for the development of GCs while completely suppressing the antibody response to surface SRBC determinants. These GCs display a near-normal DZ/LZ polar-

ization as well as normal T_{FH} and T_{FR}. Moreover, the data suggested that the GCs most probably develop in response to intra-cellular SRBC determinants as well as to the passively administered suppressive IgG. The differences in the GC response between the IgG-suppressed mice and the mice immunized with SRBC (Fig. 1-2, **paper II**) alone may be explained by the possibility that the mice react to different antigens.

Paper III

Epitope specific suppression of IgG responses by passively administered specific IgG: Evidence of epitope masking

IgG-mediated decrease of the amount of SRBC in the spleen is dependent on activating FcγRs and does not correlate with suppression.

Administration of specific IgG together with SRBC has been shown to increase antigen clearance from the blood and decrease the localization of SRBC in the MZ of the spleen (198). Whether the decreased amounts of SRBC in the spleen contributed to the suppression of antibody responses has not been directly assessed nor has the involvement of FcγRs been studied. To investigate these questions, BALB/c mice and mice deficient in FcγRI, III and IV (FcRγ KO) were immunized with $5x10^7$ NP-SRBC ± IgG anti-SRBC or with $1x10^7$ NP-SRBC alone (Fig. 1, **paper III**). We confirmed that IgG indeed caused a decrease in the antigen load of the MZ (Fig. 1A-H and Q, **paper III**) and the entire spleens (Fig. 1R, **paper III**) of the BALB/c mice, as suggested by a previous study (198). Immunization with $1x10^7$ NP-SRBC alone or IgG together with $5x10^7$ NP-SRBC resulted in comparable levels of NP-SRBC in the spleen (Fig. 1Q-R, **paper III**). When the IgG anti-SRBC response was analyzed in parallel, a completely different picture emerged (Fig. 1S, **paper III**). The BALB/c mice immunized with $1x10^7$ NP-SRBC alone generated a potent IgG anti-SRBC response, comparable to that after immunization with $5x10^7$ NP-SRBC alone, whereas mice immunized with specific IgG together with $5x10^7$ NP-SRBC had an almost completely suppressed antibody response (Fig. 1S, **paper III**). Moreover, the IgG did not decrease the amounts of SRBC in the spleens of the FcRγ KO mice but still suppressed the IgG anti-SRBC response in these mice (Fig. 1I-S, **paper III**). These findings suggested that the role of antigen clearance by specific IgG is negligible for IgG-mediated suppression. Moreover, these data demonstrated that the IgG-mediated clearance of SRBC in the spleen is dependent on activating FcγRs.

Suppression of IgG-responses is epitope specific

The specificity of IgG suppression has primarily been studied in systems analyzing IgM responses during the first week after immunization (141, 142, 159, 169, 170, 193, 194, 198, 199). It is important to address, to understand

the mechanism underlying IgG suppression. To further explore whether the suppression of antibody responses is epitope specific or non-epitope specific, C57BL/6 mice were immunized with $5x10^7$ NP-SRBC together with either IgG anti-SRBC or IgG anti-NP, or with NP-SRBC alone (Fig. 2, **paper III**). Mice were bled every two weeks, and the serum IgG-responses to SRBC and NP were analyzed by ELISA. We found that the passively administered IgG anti-SRBC exclusively suppressed the IgG anti-SRBC response but not the IgG anti-NP response (Fig. 2C-D, **paper III**). In contrast, the administration of IgG anti-NP suppressed only the IgG anti-NP response, leaving the anti-SRBC response intact (Fig. 2A-B, **paper III**). These results demonstrated that the suppression of IgG responses is strictly epitope specific under these conditions. These observations provide compelling evidence that epitope masking is the main mechanism by which IgG suppression operates.

IgG suppresses all tested pathways of B cell differentiation

Whether IgG suppresses the priming for a memory antibody response to SRBC is unclear because earlier studies have yielded conflicting results (141, 189, 201-203). Moreover, little is known about the IgG-mediated suppression of parameters of the immune response other than the early IgM response. Here, we sought to determine whether the passively administered IgG suppress the early extra-follicular plasmablasts and cells derived from the GC reaction, such as long-lived plasma cells and memory cells.

To assess whether the specific IgG suppresses the antigen-specific B cells of GC or non-GC origin, an NP-system was used. Here, C57BL/6 mice were immunized with NP-SRBC ± IgG anti-NP and their spleens were analyzed six days after immunization, through flow cytometry and confocal laser scanning microscopy (Fig. 3, **paper III**). A small population of λ_1^+ NP-binding $B220^+$ B cells could be detected through flow cytometry (Fig. 3A-C, **paper III**). This population composed 0.05% of the total $B220^+$ B cell pool and was represented in both the $GL7^+$ PNA^+ GC compartment and the non-GC compartment (Fig. 3A-C, **paper III**). Moreover, this NP-specific B cell population was not observed after immunization with native SRBC (Fig. 3C, **paper III**). The administration of IgG anti-NP completely suppressed the generation of NP-specific B cells both in the GC and non-GC compartments (Fig. 3A-C, **paper III**). By using confocal laser scanning microscopy, we demonstrated that the majority of NP-specific B cells were located in the extra-follicular foci as well as in IgD^- GC-areas of the B cell follicles, although the latter were less brightly stained by NP-PE (Fig. 3D-I, **paper III**). In this analysis, the IgG anti-NP suppressed the generation of NP-specific cells, thereby confirming the flow cytometry data (Fig. 3, **paper III**). Thus, the passive administration of specific IgG efficiently suppresses the generation of antigen-specific B cells in the spleen.

One hallmark of B cell responses is the production of antibodies by plasma cells. Therefore, we sought to determine whether the specific IgG affect the development of plasma cells in the spleen and bone marrow. BALB/c mice were immunized with SRBC ± IgG anti-SRBC, and the amounts of IgG-secreting cells were determined by ELISPOT analysis over a period of ten weeks (Fig. 4, **paper III**). The administration of specific IgG suppressed the plasma cell response in both the spleen and the bone marrow (Fig. 4, **paper III**). To study the suppression of memory induction, BALB/c mice were primed with $5x10^7$ SRBC or $5x10^6$ SRBC ± IgG anti-SRBC and boosted after ten weeks with a suboptimal dose of SRBC (Fig. 5, **paper III**). One group of naïve mice received only the booster dose without prior priming. The primary and the memory IgG anti-SRBC responses were analyzed through ELISA using end-point titers (Fig. 5, **paper III**). IgG present at priming suppressed the primary and the memory IgG anti-SRBC responses by >97%. A minor memory response was detected after boosting the IgG-suppressed mice, because these mice responded to a slightly higher extent than the mice given booster alone (Fig. 5, **paper III**).

However, if the primary antibody response was suppressed by >97%, the secondary response was also suppressed by >97% (Fig. 5, **paper III)**. Thus, the priming for memory responses was suppressed to the same degree as the primary response. Hence, it could be expected that a partially suppressed primary response would result in a partially suppressed secondary response. This relationship might also explain the occasional observation of the inefficient suppression of immunological memory induction by IgG.

These data demonstrate that IgG suppresses the generation of plasma cells in the spleen and bone marrow and that IgG present at priming suppresses the induction of immunological memory to the same extent as the primary response.

In conclusion, the data presented in **paper III** showed that the FcγR-dependent IgG-mediated antigen clearance is an inadequate explanation for the suppression of antibody responses and that the main mechanism underlying IgG suppression is most probably epitope masking. Furthermore, this is the first demonstration of the suppression of several parameters of an antibody/B cell response by specific IgG, thereby providing a more comprehensive understanding of IgG suppression.

General discussion

The suppression of erythrocyte-specific antibody responses by passively administered IgG is a well-known phenomenon that has routinely been used in clinical settings since the end of the 1960s in RhD prophylaxis to prevent HDFN. Therefore, it is of great theoretical and clinical interest to understand how IgG suppression functions. Although IgG suppression has been extensively studied for decades, the underlying mechanism has remained elusive. The focus of this thesis was to elucidate the underlying mechanism of the IgG suppression of antibody responses by using native or haptenated SRBC as antigens.

To explain how the IgG suppression functions, the following proposed mechanisms were studied: (I) The central inhibition of B cells by co-crosslinking of BCR-FcγRIIB, (II) FcγR-mediated phagocytosis and IgG-mediated antigen clearance, (III) C-mediated lysis and (IV) epitope masking. Whether IgG suppress other parameters of the immune response to SRBC was also studied.

The most important question to address in unveiling the mechanism of IgG suppression is whether the suppressive capacity is mediated by the Fc region of IgG. The observation that the suppression occurs equally well in mice lacking FcγRs or complement factors strongly suggests that the suppression is Fc independent. How can this conclusion be accommodated with the findings of non-epitope-specific suppression and the inability of the $F(ab')_2$ fragments to suppress immunological responses as shown by several groups and reviewed above?

Specificity of IgG-suppression

IgG suppression of IgM responses can be both epitope specific and non-epitope specific. We argue that this observation can be explained by the IgG bound to the antigen sterically hindering B cells from gaining access to the antigen, thereby depriving the B cells of BCR-signals. An IgG molecule masks the epitope to which it binds (thus resulting in epitope-specific suppression) and sometimes also the surrounding epitopes (thus resulting in non-epitope-specific suppression). Non-epitope-specific suppression would require that specific epitopes be present at high enough density to allow the IgG to sterically hinder B cells from binding not only to the same epitopes bound to the IgG but also to neighboring epitopes. In support of this possibility, hapten-specific IgG have been shown to require high epitope density in order to suppress non-epitope-specific IgM responses (143, 198). Whether the suppression of serum IgG responses is epitope or non-epitope specific has not been addressed previously and is part of **paper III**. We have shown that the suppression of IgG responses appears to be strictly epitope specific, on the basis of the observation that immunization with IgG anti-SRBC together with NP-SRBC suppressed only the IgG anti-SRBC response. In con-

trast, immunization with IgG anti-NP together with NP-SRBC suppressed only the IgG anti-NP response (Fig. 2, **paper III**). One could argue that there is a likelihood of achieving non-epitope-specific suppression of IgG responses on the basis of the epitope density, as mentioned above. However, this possibility seems highly unlikely, given the results obtained using IgG anti-SRBC (Fig. 2, **paper III**). In the tested conditions, the epitopes available for IgG anti-SRBC were so abundant that the IgG anti-SRBC should have been able to mask the NP-epitopes if non-epitope-specific suppression of the IgG-response were possible.

Why is the suppression of IgG responses strictly epitope specific, whereas the suppression of IgM responses can be both epitope specific and non-epitope specific (Fig. 3)? The non-epitope-specific suppression of IgM responses, observed during high density conditions, may be explained by the generally low affinity of the IgM^+ B cells, thus making them unable to compete with the passively administered high-affinity IgG and, owing to steric hindrance, preventing these B cells from binding both to specific and to non-specific epitopes. This hindrance would result in a lack of antibody responses to both types of epitopes, i.e., non-epitope-specific suppression. In contrast, the IgG^+ B cells usually express BCRs of high affinity and could therefore successfully compete with the passively administered IgG for binding to epitopes that are not directly masked by the specific IgG. This scenario would result in a response to the neighboring epitopes, i.e., epitope-specific suppression. The observation that the suppression of IgG responses is strictly epitope specific provides evidence supporting the epitope masking hypothesis. Other indirect support comes from studies demonstrating that the optimal suppression requires the IgG to bind its antigen with high affinity (143, 169, 206, 207) and with high epitope density (143, 170, 172, 193, 208) and that the suppression by monoclonal IgG additively improves when the monoclonal IgG can bind to different epitopes on the same antigen (172, 183).

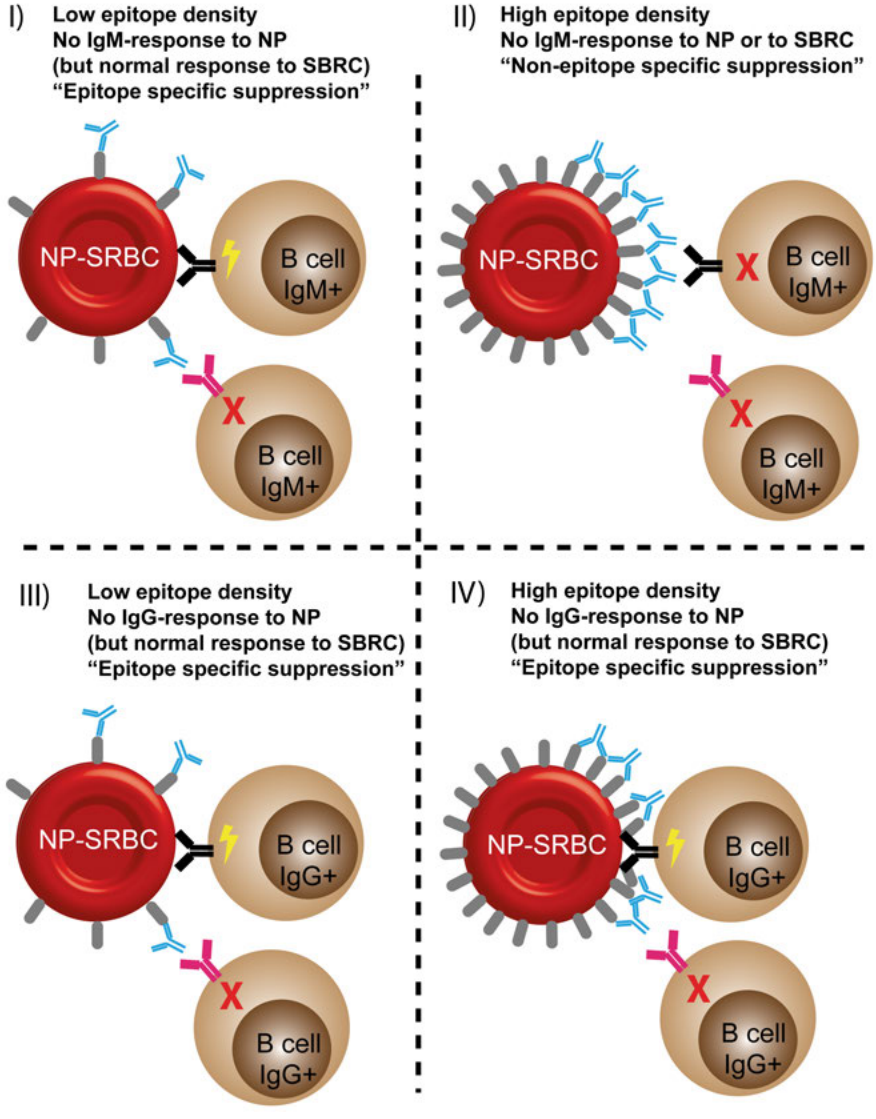

Figure 3. **Specificity of IgG-suppression.** IgG suppresses both IgM and IgG responses to the epitopes to which it binds. In addition, non-eptiope specific suppression of IgM-responses can be seen when the epitope density is high. Thus, regarding IgM-responses, low epitope density results in epitope specific suppression while high epitope density results in non-epitope specific suppression. In contrast, suppression of IgG-responses appears to be exclusively epitope specific because non-epitope specific suppression is not observed even during high epitope density conditions. For example, IgG anti-SRBC administered together with SRBC-NP suppresses IgG anti-SRBC but not IgG anti-NP (Fig. 2 **paper III**). A hypothetical explanation for this discrepancy would be that IgG^+ B cells often are of higher affinity and can more efficiently overcome the steric hindrance by suppressive IgG, bind to epitopes not blocked by IgG and thus get stimulated through their BCR. IgM^+ B cells are of low affinity and may have problems overcoming the steric hindrance.

The F(ab')$_2$ conundrum

As mentioned above, studies of IgG suppression by F(ab')$_2$ fragments have yielded conflicting results (141, 159, 169, 187-192), and it is important to understand how these discrepancies may have arisen.

How can the inability of F(ab')$_2$ to suppress be explained?

The inability of F(ab')$_2$ to suppress may be due to several reasons. First, IgG suppression operates via Fc functions. Second, F(ab')$_2$ is rapidly removed from the circulation, because it is unable to bind to FcRn, and the loss of this protection makes it more susceptible to degradation. This rapid removal of F(ab')$_2$ *in vivo* may ultimately lead to levels sufficiently low to prevent suppression of the antibody response. Therefore, it is imperative to adjust the doses of F(ab')$_2$ so that the levels *in vivo* are comparable to intact IgG during the timeframe of the experiment to draw reliable conclusions. Third, the variable antigen binding sites on the F(ab')$_2$ may be affected during their preparation by pepsin digestion. This variability should be controlled for by comparing the antigen binding capacity of the F(ab')$_2$ preparation with intact IgG by hemagglutination assays.

How can the ability of F(ab')$_2$ to suppress be explained?

Similarly, there are different interpretations of the successful suppression by F(ab')$_2$. First, IgG suppression operates independently of Fc functions. Second, the suppressive capacity of F(ab')$_2$ is simply due to the contamination with intact IgG, such that the Fc region of the intact IgG induces suppression. Therefore, it is essential to verify the purity of F(ab')$_2$ preparations by using techniques such as ELISA and SDS-PAGE. Third, a possible contamination by transforming growth factor (TGF)-β has also been suggested as an explanation of the suppressive capacity of F(ab')$_2$.

Although the considerations mentioned above were accounted for, no consensus could be reached regarding the suppressive ability of F(ab')$_2$. Thus, although theoretically it seems easy to evaluate the Fc dependence on the basis of the passive administration of F(ab')$_2$, doing so is difficult in practice. The technical difficulties discussed above will always leave this type of experiment open to criticism, and a more direct approach to test Fc dependence has been to use transgenic/KO mice.

The roles of FcγRs and C

The involvement of FcγRs in IgG suppression of IgM anti-SRBC and early IgG anti-SRBC responses was directly tested in mice lacking the activating FcγRI, III and IV (FcRγ KO), the inhibitory FcγRIIB, or FcRn (141). It has clearly been demonstrated that the suppression operates equally well in these mice and wild-type mice. This observation was cause for numerous debates at the time, because it had generally been thought that the IgG suppression

functioned via FcγRs. One of the aims of **paper I** was to confirm these findings and to investigate whether the suppression of long-term IgG responses operates independently of FcγRI, III and IV (FcRγ KO) or FcγRIIB (Fig. 3, **paper I**). We demonstrated that the suppression of IgM anti-SRBC and IgG anti-SRBC responses functioned equally well in the FcRγ KO and FcγRIIB KO mice compared with the wild-type mice (Fig. 3, **paper I**), thereby confirming and extending previous findings (141). Simultaneously, the Lazarus laboratory reported that suppression of antibody responses to allogeneic HOD-RBCs function equally well in the absence of FcγRI, III and IV or FcγRIIB (190). Interestingly, IgG also suppress antibody responses independently of FcγRIIB if they are administered several days after the antigen (140). These results, together with the ability of $F(ab')_2$, IgE (141, 190, 209) and IgM (171, 210, 211) to suppress antibody responses, strongly suggest that FcγR-mediated mechanisms cannot explain IgG suppression.

Importantly, the negative regulation by FcγRIIB *in vitro* and *in vivo* has been documented in other experimental systems (11). Deletion of FcγRIIB results in augmented antibody responses, higher risk of developing autoimmune diseases and higher sensitivity to anaphylaxis (30, 138). In addition, the enhancement of antibody responses to small proteins by passively administered specific IgG is elevated in FcγRIIB KO mice, thus further confirming the negative regulatory capacity of this receptor (138). Therefore, it is important to note that I do not refute the negative regulatory role of FcγRIIB. The inhibition by FcγRIIB requires BCR-antigen interactions and B cell activation signals. However, when the B cells do not have the opportunity to meet their specific antigens, because of the epitope masking by IgG, no ITAM signal can be generated through the BCR after antigen binding. Because FcγRIIB acts by inhibiting ITAM signaling, a negative signaling by FcγRIIB would not be initiated in this situation, therefore explaining why this receptor is dispensable in IgG suppression (Fig. 3, **paper I**).

In **paper I,** it was demonstrated that the suppression of antibody responses functions equally well in the absence of C1q, C3 or CR1/2 (Fig. 1-2, S1 fig., **paper I**). Conclusions regarding the suppression of IgG responses in these mice were difficult to draw, owing to the intrinsic impairment of antibody responses to antigens alone, but we had clear indications that IgG responses were indeed suppressed (S1 fig., **paper I**). These results suggested that IgG suppression operates independently of C-activation, thus further suggesting that Fc-dependent mechanisms cannot explain IgG suppression. In **paper I,** the possible redundancy of FcγRs and C was not assessed for several reasons. First, it is unlikely that the combination of FcγR and C deficiencies would alter the outcome, because no effect on suppression by IgG was observed when individual components were absent. Second, analyzing antibody responses in C-deficient mice is difficult, owing to the severely impaired antibody responses to the antigen alone. Therefore, it can be expected that combined KO mice, lacking multiple C components and FcγRs,

would have a very poor antibody response to the antigen alone, thus making the analysis of suppression technically impossible. Thus, to date there is no direct evidence for the involvement of FcγRs and C in IgG suppression *in vivo*. Fc dependence has been indirectly inferred by the lack of suppression with $F(ab')_2$ fragments and non-epitope-specific suppression. In contrast, there is abundant compelling evidence showing that IgG suppression is Fc independent, and that non-epitope-specific suppression can be reconciled with Fc-independent mechanisms such as epitope masking.

The contribution of antigen clearance

It has been demonstrated that IgG increases the clearance of SRBC from the blood and decreases the localization of the antigen to the spleen (198). The contribution of antigen clearance by IgG to the antibody response has not previously been directly tested. In **paper III**, the IgG-mediated clearance was assessed in parallel with antibody responses, and we clearly showed that the clearance in the spleen is dependent on activating FcγR and that it does not correlate with the suppression of antibody responses (Fig. 1, **paper III**). Moreover, in a model using HOD-RBCs as the antigen, monoclonal anti-OVA IgG suppressed the OVA-specific antibody response regardless of whether clearance was induced (142). These observations are difficult to reconcile with IgG-mediated antigen clearance as a major explanation for IgG-suppression. Moreover, if clearance were the dominant mechanism, the IgG would be expected to suppress the priming of $CD4^+$ T cells, whereas epitope masking would allow for T cell priming. Indeed, the $CD4^+$ T cell priming was almost normal in the IgG-suppressed mice (141, 198, 204). Together with the results presented in **paper III,** these observations suggest that the IgG-mediated clearance is inadequate to describe the suppression of antibody responses.

Epitope masking as the dominant mechanism behind IgG-suppression

Epitope masking is difficult to study directly and has always been based on the exclusion of other possibilities. However, it is compatible with the majority of experimental findings listed below. In **paper III** we showed that IgG suppresses many parameters of an antibody/B cell response to SRBC. This observation can be reconciled with epitope masking, because the logical outcome of depriving the B cells of the ability to interact with their specific antigen is the lack of B cell activation and further differentiation into different effector cells. The additional indirect support for epitope masking provided by **paper II** was that the IgG-suppressed mice had severely suppressed antibody responses to surface SRBC determinants while mounting a GC response, presumably to the intra-cellular SRBC determinants and to the suppressive IgG. These observations may be explained by the efficient masking of surface epitopes by the passively administered IgG, thereby leaving only arrays of IgG/Fc and intra-cellular SRBC epitopes available for

recognition by the B cells. Together, these observations provide compelling evidence that epitope masking is the dominant mechanism underlying IgG suppression. To the best of our knowledge, these results showing that the suppression of IgG responses is strictly epitope specific provide the most direct proof to date of this hypothesis (Fig. 2, **paper III**).

Findings compatible with epitope masking:

- Independence of FcγRs and C
- IgG suppresses antibody responses independently of FcγRIIB
- Suppressive ability of $F(ab')_2$, IgM and IgE
- The non-epitope-specific suppression of IgM responses being dependent on high epitope density
- Strictly epitope-specific suppression of IgG responses
- Lack of correlation between antigen clearance and suppression of antibody responses
- Correlation of suppression with the affinity of the passively administered IgG and epitope density
- Additive effects of mixtures of monoclonal antibodies
- Lack of suppression of $CD4^+$ T cells

Findings incompatible with epitope masking:

- The inability of $F(ab')_2$ to suppress

Relevance to RhD-prophylaxis

Whether murine models of IgG suppression reflect the situation in RhD prophylaxis is a matter of controversy. Two major arguments have been proposed criticizing the view that the IgG-mediated suppression of SRBC responses in mice is a relevant model for IgG-mediated suppression of anti-RhD responses in RhD^- women. First, xenogeneic SRBC have been claimed not to mimic the situation elicited by allogeneic RhD^+ RBCs. Second, it has been shown that low doses of passively administered IgG anti-RhD do not bind to all RhD-epitopes on RhD^+ erythrocytes, thus making it difficult to reconcile RhD prophylaxis with epitope masking.

To address the first issue, I would argue that SRBC are indeed good model antigens simply because of their high immunogenicity. Immunization with SRBC can be expected to elicit a more potent immune response than alloimmunization with RhD^+ RBC. Therefore, it would be more difficult to suppress the SRBC response compared with the RhD-response, and minute amounts of IgG antibodies could efficiently suppress both responses. Moreover, data to date regarding the suppression using allogeneic murine HOD-RBCs have been compatible with the results obtained using SRBC.

To address the second issue, I would argue that it is indeed possible that epitope masking may contribute to the suppression by IgG anti-RhD, although IgG anti-RhD suppresses the antibody response even at doses too low

to directly cover all RhD epitopes (182). One parameter that has not been considered is the possibility that IgG anti-RhD may indirectly cover the neighboring RhD epitopes by steric hindrance. This partial masking may be sufficient to suppress the weak response to allogeneic RhD^+ RBCs. In line with this possibility, IgG anti-Kell suppresses responses to RhD (212), and these antigens are located close to each other on the RBC surface (213). Moreover, RhD is not uniformly distributed on the RBC surface (214). These observations might make it possible for epitope masking to contribute to the suppressive effect of anti-RhD by steric hindrance and should not be overlooked.

It cannot be excluded that antigen clearance contributes to the suppression observed in RhD prophylaxis. However, human monoclonal anti-RhD antibodies can suppress without inducing clearance and induce clearance without suppressing the immune response. Therefore, it seems disputable whether clearance plays an important role (174, 200). To improve the efficacy of monoclonal anti-RhD, on the basis of studies in murine models, it may be beneficial to optimize these antibodies regarding antigen binding properties rather than their capacity to activate FcγRs or induce clearance. Antibodies with varying binding specificities may also enable the direct testing of the contribution of epitope masking in RhD prophylaxis, e.g., by assessing whether mixtures of monoclonal anti-RhD antibodies have additive effects.

Concluding remarks

In summary, the data presented in this thesis contribute to unveiling the masked mechanism underlying IgG-mediated suppression of antibody responses:

- The suppression of IgM and long-term IgG responses occurs independently of FcγRs and C.
- GC responses occur in response to intra-cellular SRBC epitopes and suppressive IgG, whereas the response to surface SRBC epitopes is completely suppressed.
- IgG-mediated antigen clearance is negligible in the suppression of antibody responses.
- IgG suppresses all tested parameters of an antibody or the B cell response.
- The suppression of IgG responses is strictly epitope specific.

These findings are difficult to reconcile with either Fc-dependent functions or antigen clearance as the main mechanisms underlying IgG suppression. Moreover, the key observation showing that the suppression of IgG responses is strictly epitope specific provides direct evidence supporting the epitope masking hypothesis.

Summary in Swedish

Om kroppen utsätts för en infektion eller ett främmande ämne, så svarar immunsystemet på detta med att bland annat producera antikroppar. Antikroppar, som är en del av förvärvad immunitet, hjälper till att minska infektioner och främmande ämnen med hög effektivitet och specificitet. Antikroppar är Y-formade molekyler, som bland annat finns i blodcirkulationen, vilka bildas av specialiserade immunceller, B lymfocyter. Antikroppar har möjligheten att känna igen särskilda strukturer, så kallade antigen, på t.ex. bakterier och andra molekyler. Denna specificitet hos antikroppen är lokaliserad till de två ”armarna” på antikroppen. När en antikropp har bundit sitt antigen kan den förmedla detta vidare till resten av immunsystemet med hjälp av ”svansen”, Fc-delen på antikroppen. Fc-delen kan binda till olika receptorer och genom denna bindning så utlöser antikroppen olika funktioner, som ser till att antigenet elimineras. Ett intressant fenomen är att antikroppar kan reglera sin egen tillverkning. Om man injicerar antikroppar tillsammans med antigenet, de binder till, i möss så kan man antingen få ett mycket starkare eller ett helt undertryckt, så kallat suppressat, antikroppssvar mot antigenet. Om höjning eller suppression sker är beroende av vilken typ av antikropp och vilket antigen, som används.

Det mest välkända exemplet är funktionen hos IgG-antikroppar är att totalt suppressa antikroppssvaret mot röda blodkroppar, erytrocyter.
Det har används sedan 1960-talet i Rhesus profylax för att förhindra hemolytisk sjukdom hos foster och nyfödda. Problematiken uppstår när en Rhesus negativ (Rh^-) kvinna bär på ett Rhesus positivt (Rh^+) foster. Vid t.ex. förlossning kan fostrets erytrocyter läcka över till kvinnan över moderkakan. Eftersom kvinnan är Rh^- så känns fostrets Rh^+ erytrocyter igen, som ett främmande ämne, och en immunreaktion hos kvinnan startar. Under nästa graviditet kommer kvinnans immunsystem att attackera fostrets Rh^+ erytrocyter och förstöra dessa, vilket leder till hemolytisk sjukdom hos fostret och den nyfödda. Rh-immuniseringen kan man förhindra genom att injicera kvinnan med IgG-antikroppar, som binder till Rh, upp till 72 timmar efter förlossningen och dessa hämmar då kvinnans immunsvar mot Rh. Denna behandling är väldigt framgångsrik och har minskat förekomsten av hemolytisk sjukdom hos foster och nyfödda med 90%.

IgG-suppression har studerats i årtionden och än så länge har man inte kunnat förklara hur IgG-antikroppar suppressar antikroppssvaret mot erytrocyter.

Målet med min avhandling var att undersöka mekanismen bakom IgG-suppression i musmodeller med erytrocyter från får (SRBC) som antigen. De flesta tidigare studier av mekanismen bakom IgG-suppression har vanligen studerat det tidiga antikroppssvaret i mjälten. Man vet mycket mindre om IgG-suppression av det långvariga antikroppssvaret i blodcirkulationen och vilka celltyper som är suppressade. En populär förklaring till IgG-suppression var att IgG aktiverade olika receptorer med sin Fc-del och att det orsakade suppressionen.

I det första delarbetet kunde vi tydligt visa att IgG suppressar både det tidiga antikroppssvaret i mjälten samt det långvariga svaret i blodcirkulationen oberoende av komponenter som kan aktiveras av Fc-delen. Tillsammans med tidigare studier, så visar det att suppressionen inte orsakas av Fc-delen på IgG. I nästa delarbete undersökte vi om IgG kan påverka utvecklingen av germinala centran (GC) i mjälten. Dessa strukturer i mjälten är nödvändiga för att producera ett antikroppssvar av hög kvalité och kvantitet. Intressant nog så kunde vi se att GC utvecklades i möss där antikroppssvaret är helt suppressat av IgG. Det visade sig senare vara så att GC bildades som svar mot de injicerade IgG-antikropparna och inte mot SRBC, antigenet i fråga. Hur kan man förklara detta? Om IgG sitter bundet på SRBC kommer de att maskera SRBC och det enda som immunsystemet kan känna igen är IgG.

Två mekanismer som är förenliga med majoriteten av studier är att IgG suppressar genom att eliminera eller att maskera antigenet från immunsystemet via så kallad epitopmaskering. I det tredje delarbetet undersökte vi hur eliminering och epitopmaskering bidrar till suppressionen av antikroppssvaret. Vi undersökte även vilka parametrar av immunsvaret mot SRBC, som kan suppressas av IgG. Vi kunde visa att suppressionen av antikroppsvaret var oförändrad oavsett om eliminering av SRBC skedde eller inte. Vi kunde även visa att IgG suppressar alla parametrar av immunsvaret mot SRBC, som vi kunde mäta. Utöver detta så kunde vi se att IgG enbart suppressar det långvariga antikroppssvaret mot de strukturer IgG binder till, vilket är det närmaste vi har kommit till ett direkt bevis för att epitopmaskering troligen är den huvudsakliga mekanismen bakom IgG-suppression.

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