Antibody Feedback Regulation and T Cells

FREDRIK CARLSSON
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, March 30, 2007 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Antibodies, passively administered or actively produced, regulate immune responses to the antigen they recognize. This phenomenon is called antibody-mediated feedback regulation. Feedback regulation can be positive or negative, resulting in >1000-fold enhancement or >99% suppression of the specific antibody response. The outcome depends on size, structure, dose, and route of administration of the antigen as well as on class and subclass of the regulating antibody. This thesis investigates the role of T cells in antibody-mediated feedback enhancement, using both in vivo and in vitro approaches. IgE-antibodies enhance antibody responses to small soluble proteins. This effect is entirely dependent on the low-affinity receptor for IgE, CD23, and most likely depends on increased antigen presentation by CD23+ B cells. Strengthening this hypothesis, we show that IgE-mediated CD4+ T cell proliferation in vitro required the presence of CD19+ CD43+ CD23+ B cells. CD23 has also been shown to negatively regulate immune responses. Transgenic mice overexpressing CD23 are known to have impaired responses to antigens in alum. We here demonstrate that they are normal regarding IgE-mediated enhancement. IgG3 enhances antibody responses, and previous data suggested involvement of complement. We found that IgG3-mediated enhancement works well in mice lacking the only Fc-receptor known to bind IgG3, CD64. Although IgG3 could enhance antibody responses it had no major effect on T cell responses. Complement-receptors 1/2 (CR1/2) are required for the initiation of normal antibody responses. Although mice lacking CR1/2 had impaired antibody responses after immunization with sheep erythrocytes, their specific T cell responses were unaffected. The presented data do not support the idea that increased complement-mediated antigen presentation is a major mechanism behind the involvement of complement in antibody responses. They support the hypothesis that antigens forming complement-containing immune complexes may activate specific B cells by co-crosslinking BCR and CR1/2.

Keywords: Fc receptors, B cell, T cell, antigen presentation, complement receptors, complement, IgE, IgG3, CD23, antibody

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ISSN 1651-6206
urn:nbn:se:uu:diva-7631 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7631)
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List of Papers

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

I Carlsson F, Hjelm F, Conrad DH, Heyman B.
IgE enhances specific antibody and T cell responses in mice overexpressing CD23.
Manuscript

II Hjelm F, Carlsson F, Verbeek S, Heyman B.
IgG3-mediated enhancement of the antibody response is normal in FcγRI-deficient mice.

III Carlsson F, Getahun A, Heyman B.
Impaired antibody responses but normal CD4⁺ T cell responses in mice lacking complement-receptors 1 and 2.
Manuscript

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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>complement</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD23Tg</td>
<td>transgenic mouse strain overexpressing CD23</td>
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<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CVF</td>
<td>cobra venom factor</td>
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<tr>
<td>DNP</td>
<td>2,4-dinitrophenyl</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment crystallizable</td>
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<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
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<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FcγR_common</td>
<td>common gamma chain</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>HEL</td>
<td>hen egg lysozyme</td>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MBL</td>
<td>mannan-binding lectin</td>
</tr>
<tr>
<td>NIP</td>
<td>4-hydroxy-3-iodo-5-nitro-phenylacetyl</td>
</tr>
<tr>
<td>NS</td>
<td>not significant</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>RhD</td>
<td>rhesus antigen D</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeat</td>
</tr>
<tr>
<td>SRBC</td>
<td>sheep red blood cell</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TD</td>
<td>thymus-dependent</td>
</tr>
<tr>
<td>TI</td>
<td>thymus-independent</td>
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TNP     2,4,6-trinitrophenyl
T_{H}   T helper cell
wt      wild-type
Introduction

Feedback regulation

Antibodies are extremely efficient in regulating the antibody response against the antigen they recognize, a phenomenon known as antibody feedback regulation (reviewed in [1-4]). The regulatory effects of passively administered or actively produced antibodies can be dramatic and may range from an almost complete suppression to a thousand-fold enhancement of antibody production, compared to the response to the antigen alone. Antibody-mediated suppression and enhancement is always antigen-specific and depends on factors such as antibody class, antibody affinity, the nature and dose of the antigen, antigen:antibody-ratio as well as the administration route of antigen and antibody.

Antibody-mediated suppression of antibody responses

Suppression of antibody responses occurs mainly towards particulate antigens such as erythrocytes (reviewed in [3-5]). Immunoglobulin (Ig) G can efficiently suppress (>99%) of an antibody response [6-8]. This property of IgG has been known for a long time and is used clinically to prevent Rhesus D antigen negative (RhD-) mothers from becoming immunized against fetal RhD+ erythrocytes acquired via transplacental hemorrhage during pregnancy or in association with delivery. At least three different mechanisms behind IgG-mediated suppression have been proposed [3]: (a) Fc-gamma-receptor positive (FcγR+) phagocytic cells effectively dispose of IgG/antigen-complexes before specific B cells are activated, (b) IgG/antigen-complexes inhibit the activation of antigen-specific B cells by cross-linking the B cell receptor (BCR) and the inhibitory FcγRIIB on the B cell surface, and (c) the antigen-specific IgG masks the epitopes of the antigen, preventing B cells from recognizing it. Whereas the first two mechanisms would be dependent on FcγRs, the latter would be independent of FcγRs. A strong argument for IgG-mediated suppression being Fc-independent, is the ability of IgG to suppress antibody responses in mice that lack all known FcγRs [7, 9]. In addition to this, F(ab')2, fragments have been shown to be able to suppress [7, 10, 11]. Other observations compatible with the epitope masking mechanism are studies showing that both IgM [8, 12] and IgE [7,
under certain conditions are able to suppress antibody responses to erythrocytes. Also, the requirement for IgG to bind antigen with high affinity [8, 14-16] and at a high density [6, 16-19] is compatible with epitope masking. The idea that suppression is Fc-dependent mainly relies on two observations. First, IgG-mediated suppression is, in certain experimental systems, non-epitope-specific [6, 8, 16, 20-23]. Second, and in contrast to observations mentioned earlier, some in vitro and in vivo studies show poor suppression by F(ab')2 fragments [8, 22, 24-26].

Antibody-mediated enhancement of antibody responses

IgE, IgM and all subclasses of IgG can enhance antibody responses (reviewed in [3]). Since the molecular mechanisms behind antibody-mediated enhancement differ depending on the antibody class and even subclass used, the antibody classes IgM, IgG and IgE will be discussed separately and in more detail below.

Enhancement via IgE

Immunization with an immune complex consisting of IgE antibody and a soluble antigen will lead to a several hundred-fold enhancement of the antibody response to this antigen compared to immunization with antigen alone. Enhanced serum levels of IgM, IgG1, IgG2a, and IgE as well as memory induction [27-30] have been demonstrated after immunization with IgE-complexed antigen. Enhancement is completely dependent on the expression of the low-affinity receptor for IgE, designated cluster of differentiation (CD) 23, since IgE is unable to enhance in mice lacking CD23 [27, 29] or in mice treated with anti-CD23 monoclonal antibodies (mAbs) [28, 30]. Any involvement of the high-affinity receptor for IgE, FceRI, has been ruled out, since IgE-mediated enhancement is (a) normal in mice lacking the common gamma chain, FcγRI [31], as these mice cannot express FceRI [31], and (b) absent in mice lacking CD23 but retaining FceRI [27, 29].

The proposed mechanism behind IgE-mediated enhancement

IgE-mediated enhancement is normal in mice lacking interleukin (IL) 4 [32]. Since IL-4 is required for expression of the CD23b isoform [33], this suggests that only constitutively expressed CD23a, found on B cells and/or on follicular dendritic cells (FDCs), is involved in IgE-mediated enhancement. FDCs can trap and retain immune complexes [34]. Therefore,
the mechanism behind IgE-mediated enhancement may be an increase of effective antigen concentration on the surface of CD23-expressing FDC in germinal centers. However, it was shown in an adoptive transfer system that CD23 has to be expressed on a bone marrow- (BM-) derived cell in order for IgE to enhance antibody responses [29]. In this study, IgE-mediated enhancement was restored when CD23+ BM cells were adoptively transferred into irradiated CD23−/− mice. No enhancement was seen when CD23−/− BM cells were transferred into irradiated wt mice. In other words, expression of CD23 on irradiation-resistant FDC in recipient wt mice did not support IgE-mediated enhancement. The most likely effector cell would therefore be the CD23+ B cell.

There are two possible, not mutually exclusive, mechanisms for how the B cell could enhance antibody responses via IgE immune complexes. (a) Co-crosslinking of CD23 and the BCR on an antigen-specific, CD23-expressing B cell could initiate a signaling cascade activating the specific B cell in question. This mechanism is unlikely, since co-crosslinking of BCR and CD23 can have a negative effect on B cell activation [35] and since IgE-mediated enhancement is absent in nude mice, suggesting involvement of T cells [28]. (b) The other possible mechanism is efficient uptake of IgE-complexed antigen via CD23 on B cells, followed by processing and presentation to antigen-specific T helper (T_H) cells. These T_H cells may in turn provide better help for specific B cells (Figure 1).

Figure 1. The most likely mechanism for IgE-mediated enhancement is the uptake of IgE-complexed antigen via CD23 on non-specific B cells, leading to increased antigen presentation and better T cell help to specific B cells. Reprinted, with permission, from [2].
In vitro studies using murine splenocytes [36], a murine B-cell hybridoma [37], and human Epstein-Barr virus (EBV)-transformed B cells [38-40] as antigen presenting cells (APCs), showed that IgE-complexed antigen induce activation of T cells more efficiently than non-complexed antigen, in a CD23-dependent manner. Increased T cell proliferation or IL-2 production in these studies was most likely due to increased antigen presentation. Furthermore, recent studies in our lab using the DO11.10 adoptive transfer system suggest that increased antigen presentation is a likely mechanism also in vivo [41]. In this study, BALB/c mice were adoptively transferred with CD4+ T cells from DO11.10 mice, carrying a transgenic T cell receptor specific for the peptide ovalbumin (OVA)323-339 presented on I-A<sup>d</sup> MHC class II. The next day, the mice were immunized intravenously (i.v.) either with OVA-2,4,6-trinitrophenyl (TNP) together with IgE-anti-TNP, or with OVA-TNP alone. T cell expansion, as measured by total number of OVA-specific transgenic CD4+ cells in the spleen three days later, was markedly increased in mice immunized with IgE/antigen and was dependent on the presence of CD23+ B cells.

CD23 – the low-affinity receptor for IgE

CD23 is the only known Fc receptor that is not a member of the immunoglobulin gene superfamily. Two major CD23 splice forms (a and b) have been described in man [42]. Structurally, the two isoforms differ only by the first N-terminal amino acids of the intracytoplasmic region. Murine B cells and FDCs constitutively express the CD23a isoform [34, 43]. The existence of a murine CD23b isoform has been uncertain, but the receptor was recently found on enterocytes in sensitized mice [44] or on enterocytes in vitro following IL-4-induction [45]. Also, murine Langerhans cells can express CD23 under certain culture conditions [46].

CD23 trimerizes on the cell surface by forming an α-helical coiled-coil “stalk” [47]. The stalk is susceptible to proteolytic cleavage [48], which leads to the shedding of soluble CD23 (sCD23). Human sCD23 may still bind IgE, but in the murine system sCD23 loses its IgE-binding ability as a result of poor trimerization following cleavage. Another difference is the ability of human CD23 [49], but not murine [50], to bind CD21. Human CD23 mediates endocytosis [51] and in functional studies using the human isoforms of CD23, it was shown that only CD23a-expressing cells mediated endocytosis. [52]
A suppressive property of CD23

CD23 is also able to downregulate antibody responses. This has been observed in different experimental systems, both as suppressed responses when CD23 is overexpressed, and as enhanced responses when CD23 is absent or non-functional. Suppressed antibody responses were first seen in two transgenic mouse strains overexpressing CD23 mainly on T cells, as a result of a Thy1.1 promoter construct [53]. These mice showed strong suppression of IgE-responses as well as diminished IgG1-responses following three different treatments (described below) known to promote IgE-responses. Five years later, another transgenic mouse strain overexpressing CD23 using the class I promoter/Ig enhancer was constructed in the Conrad-lab [54]. These CD23-transgenic (henceforth referred to as CD23Tg) mice overexpressed CD23 on resting and activated B- and T-lymphocytes [54] as well as on FDCs [55]. CD23Tg mice were compared to wt mice [54] following these IgE-promoting treatments:

- Mice were immunized and boosted 14 days later subcutaneously (s.c.) with keyhole limpet hemocyanine coupled to 2,4-dinitrophenyl (KLH-DNP) suspended in alum containing heat-killed Bordetella pertussis. Total serum IgE- and antigen-specific IgG1-levels in antigen/alum-immunized mice were assayed.
- Mice were infected s.c. and boosted 20 days later with Nippostrongylus brasiliensis larvae, and serum levels of total IgE and specific IgG1 were followed over time.
- Mice were immunized with anti-IgD-antibodies i.v. and assayed for total serum IgE as well as for antibody-forming cells.

CD23Tg animals consistently had suppressed serum levels of IgE compared to wt animals following all three treatments. It was shown that decreased serum IgE was not due to decreased half-life of IgE. Suppression of IgG1 was also observed in transgenic animals, although this effect was less consistent. In addition, the number of antibody-forming cells was decreased in transgenic animals [54]. In an adoptive transfer study of CD23Tg animals, it was demonstrated that the cell important for the suppressive effects was a non-lymphoid, irradiation-resistant cell, i.e. most likely the FDC [55].

Analogous to the studies of transgenic animals, a reduction in IgE-and IgG1-titers were also seen when wt mice were treated with an inhibitor of proteolytic processing, leading to CD23 overexpression [56]. Conversely, CD23<sup>-/-</sup> mice produced elevated titers of antigen-specific as well as total IgE compared to wt mice [57]. Enhanced responses in the absence of a functional CD23 molecule were also shown using the New Zealand Black mouse strain. These mice carry a variant CD23 allele making the receptor unable to
trimerize on the cell surface, leading to low affinity for IgE and to a CD23-/-
like phenotype with elevated IgE- and IgG1-responses to antigen
precipitated in alum [58].

Enhancement via IgG1, IgG2a and IgG2b

IgG1, IgG2a, and IgG2b efficiently enhance antibody responses to soluble
protein antigens (reviewed in [3, 59]). Enhancement by IgG1, IgG2a, and
IgG2b antibodies works in the absence of complement activation or
complement receptor (CR) 1/2 [60, 61]. Some Fc receptors contain the γ
subunit, FcRγ. This subunit is required for the proper assembly and function
of the Fc-receptors and contains an immunoreceptor tyrosine-based
activation motif, ITAM, responsible for intracellular signaling during
receptor crosslinking. ITAM-motifs are also responsible for activating signal
transduction through other receptors such as the BCR and the T cell receptor
(TCR). Mice deficient of FcRγ lack the activating Fc-receptors FcγRI
(CD64), FcγRIII (CD16), FceRI [62] as well as the newly discovered
FcγRIIV [63]. In FcγRII-/- mice, IgG1 and IgG2a can not enhance antibody
responses, and enhancement via IgG2b is reduced at early timepoints [31],
suggesting that enhancement via these antibody subclasses is dependent on
activating FcγRs. In vitro, dendritic cells and macrophages, both expressing
activating FcγRs, efficiently present antigenic peptides from IgG/antigen
complexes to T cells [64-66], and it is likely that this mechanism explains
IgG-mediated enhancement of antibody responses in vivo. Findings
supporting this were recently obtained from an adoptive transfer model [67].
In this study, CD4+ cells from DO11.10-mice, were adoptively transferred
i.v. to wt (BALB/c) mice and to FcRγ-/- mice. The following day, animals
were immunized i.v. with OVA-TNP or with IgG2a-anti-TNP + OVA-TNP.
Increased IgG2a-mediated OVA-specific T cell proliferation followed by an
increased IgG-anti-OVA production was seen in wt but not in FcRγ-/-
animals.

The only known inhibitory FcγR in mice, FcγRIIB, is expressed on all
hematopoietic cells except erythrocytes, T cells and natural killer cells [68].
FcγRIIB contains an immunoreceptor tyrosine-based inhibitory motif, ITIM.
When cocrossliking of FcγRIIB and a receptor containing an ITAM-motif
occurs, the signal from the ITAM is inhibited by the ITIM. FcγRIIB can in
this way negatively regulate many processes. It was recently shown to be
involved in modulating IgG-mediated enhancement, as mice lacking
FcγRIIB had an “enhanced” IgG1-, IgG2a- and IgG2b-mediated
enhancement compared to wt mice [31, 67]. FcγRIIB-/- mice generally show
augmented antibody responses and anaphylactic responses [69].
The complement system

The complement system is an important mediator of both innate and acquired immunity. Complement can participate in the regulation of antibody responses and aid in the clearance of immune complexes. Complement also opsonises bacteria for enhanced phagocytosis, contributes to inflammatory responses and lyses bacteria. The complement system consists of about 30 serum proteins that can be divided into four different cascade-like series of events, or pathways (schematically outlined in Figure 2). The classical pathway, the alternative pathway and the lectin pathway provide three alternative routes to the cleavage of C3 – the central event in complement activation. In all three of these complement-activating pathways, C3 cleavage then leads to C5 cleavage, which initiates the fourth pathway, called the lytic pathway. The lytic pathway ends in the formation of a multimolecular structure called the membrane attack complex (MAC). The MAC forms transmembrane channels on the surface of the target cells, which lead to their destruction by lysis.

The classical pathway

The classical pathway connects the adaptive immune system to complement activation. This route of activation is primarily dependent on the preceding binding of IgM or IgG-antibodies to the surface of the target cell. C1, a multi-subunit protein containing three different proteins (C1q, C1r and C1s), then binds to the Fc region of these antibodies. C1 binding does not occur to antibodies that have not complexed with antigen, since the C1q subunits must crosslink at least two antibody molecules (two IgG antibodies or one conformationally changed IgM pentamer) before a stable activated C1 is firmly fixed. Activated C1 will then cleave C4 into C4a and C4b. The C4b fragment binds to the membrane, and the C4a fragment is released into the microenvironment. Activated C1 also cleaves C2 into C2a and C2b. C2b binds to the membrane in association with C4b, and C2a is released into the microenvironment. The resulting C4bC2b complex is a C3 convertase, which cleaves C3 into C3a and C3b. While C3a is released into the microenvironment, C3b binds to the membrane in association with the C4bC2a complex. The new C4bC2bC3b complex is a C5 convertase. The classical pathway ends with this generation of C5 convertase.
Figure 2. The complement system.
The lectin pathway

The lectin pathway is initiated by the binding of mannose-binding lectin (MBL) to carbohydrate groups displayed on the surfaces of a wide range of microorganisms. Binding of MBL to a pathogen results in the association of two serine proteases, MASP-1 and MASP-2. Formation of the MBL/MASP-1/MASP-2 tri-molecular complex results in the activation of the MASPs and subsequent cleavage of C4 into C4a and C4b. MASP-I and MASP-II are very similar to the C1r and C1s molecules of the classical pathway. Activated MASPs cleave C4 into C4a and C4b as well as C2 into C2a and C2b. As in the classical pathway, this will first lead to the formation of the C3 convertase C4bC2a and subsequently to the formation of the C5 convertase C4bC2aC3b.

The alternative pathway

Activation of the alternative pathway does not require antibody binding to the target surface. In plasma, C3b is continuously produced due to spontaneous conversion of C3 to C3b. Normally C3b is quickly inactivated by hydrolysis, but may be deposited onto microbial surfaces where it will be stabilized by binding of factor B. Factor D then cleaves factor B into Bb, which remains bound to C3b, and the smaller fragment Ba, which is released into the microenvironment. Remaining on the microbe surface is the C3bBb complex, which is the C3 convertase of the alternative pathway. The C3 convertase quickly gives rise to more C3b, thus setting up an amplification sequence. Some of the C3b will bind to the C3 convertase itself and form C3bBbC3b, a C5 convertase. The generation of C5 convertase is the end of the alternative pathway.

The complement system and antibody responses

**Complement defects lead to impaired humoral responses**

The complement system is essential for the generation of a normal antibody response in mammals (reviewed in [70]). This first became clear in the early 1970s when Pepys showed that mice depleted of C3 by treatment with cobra venom factor (CVF) had lower antibody responses than normal mice [71]. Later it was shown that humans [72], guinea pigs [73-75], and dogs [76] with genetic deficiencies in complement factors C2, C3, or C4, had impaired antibody responses. Similarly, knockout mice lacking C1q [77], C3 [78], or C4 [78] had weaker antibody responses than normal mice. Mice defective in factor B [79] showed no obvious phenotypic difference from wt mice,
suggesting minor contribution by the alternative pathway. Since “normal” C5-deficient mouse strains, such as AKR, show normal antibody responses and since involvement of MBL is not likely (Jens Jensenius, personal communication), neither the lytic pathway nor the lectin pathway seem to play a major role in sustaining normal antibody responses.

Two receptors for activated products of the C3 and C4 components, CR1 and CR2 (Figure 3), have been implied in the initiation and regulation of the immune response by antigen trapping, B cell activation, and isotype switching. In the murine system, CR1 (CD35, 190 kDa [80] and CR2 (CD21, 150 kDa [81]) are the alternatively spliced products of a common gene, designated Cr2 [82]. The extracellular parts of CR1 and CR2 consist of repeating amino acid elements called short consensus repeats (SCRs). CR1/2 differ in their ligand-binding specificities as the 15 SCRs long CR2 binds iC3b, C3d,g, C3d and C4d, whereas CR1 (21 SCRs) in addition to these also binds C3b and C4b.

![Figure 3. Structures and ligand-binding sites of murine CR1/2.](image)

Mice lacking the Cr2-gene have severely impaired antibody responses [83-87]. Because of the lack of both CR1 and CR2 in Cr2⁻/⁻ mice, there have been difficulties in determining which of the two receptors are needed for a normal humoral response. Nevertheless, in many studies CR2 has been shown to play a major role. MAbs blocking CR1 and CR2 (mAb 7G6), but not mAbs blocking CR1 alone (mAb 8C12), inhibit antibody responses [88]. A murine, recominant soluble CR2 competed for ligand with cellular CR2 and inhibited antibody responses [89]. Cr2⁻/⁻ mice reconstituted with human
transgenic CR2 had normal antibody responses [90]. Furthermore, in the human system where CR1 and CR2 are transcribed from separate genes, increased B cell signaling is seen during BCR/CR2-crosslinking but not during BCR/CR1-crosslinking [91, 92], supporting a role for CR2.

**Is the B cell or the FDC most important?**

In the murine system, primarily B cells and FDCs express CR1/2 [93]. However, whether a normal antibody response requires the expression of CR1/2 on the B cell or on the FDC is still unclear. Some reports show an important role for the FDC [94], while other studies favor the B cell as the effector cell [85, 87]. CR2 is associated with trapping of immune complexes on FDC in germinal centers and, expressed by the B cell, it also acts as part of the coreceptor for B cell activation together with CD19/TAPA1/CD81. The B cell coreceptor complex enhances the activation signal from the BCR during crosslinking [92, 95, 96] and thus lowers the threshold for B cell activation. A recombinant model antigen, hen egg lysozyme (HEL), fused to several units of murine C3d, was up to a 10,000-fold more immunogenic than HEL alone. [97]

**Three possible ways in which complement may be involved in the generation of antibody responses**

Why an intact classical complement pathway is needed for normal antibody responses is not completely understood. CR2, expressed on B cells and FDCs, seems to play a major role. At least three different, not mutually exclusive, mechanisms have been suggested:

- Immune complexes (antigen + antibody + complement) spontaneously formed *in vivo* are captured by, and enriched on, FDCs (via CR2) where they are recognized by specific B cells.
- Antigen-specific B cells need the BCR-signal plus a second signal via the B-cell co-receptor (CR2/CD19/TAPA-1) in order to be activated, and immune complexes co-crosslink BCR and CR2 (Figure 4).
- Non-specific B cells capture immune complexes via CR2 and present peptides to antigen-specific T cells, which in turn give help to antigen-specific B cells.

Whereas the first two mechanisms would be independent of T cell help, the third mechanism would be dependent on antigen presentation and help from antigen-specific T cells.

**CR2 in antigen presentation**

Thus, one possible explanation for the requirement of CR1/2 on B cells is that complement-containing immune complexes could be endocytosed via CR1/2 on B cells (without any interactions with the BCR) and presented to
specific CD4\(^+\) T cells. There is ample evidence from \textit{in vitro} studies that B cells can indeed endocytose complement-containing immune complexes via CR1/2 and present antigenic peptides to T cells [98-104]. Conclusions from \textit{ex vivo} systems vary, and in one study T cell priming was impaired in C3\(^{-/-}\) mice [105], whereas in another report T cell priming in C3\(^{-/-}\) and Cr2\(^{-/-}\) mice was normal although antibody responses were impaired [106]. In spite of the overwhelming evidence that CR2 is involved in antigen presentation \textit{in vitro}, it is not clear whether this mechanism also operates \textit{in vivo}. Perhaps owing to the difficulty in assessing specific T cell responses \textit{in vivo}, to our knowledge only one report has addressed this question. Using the hapten-carrier approach in an adoptive transfer system, we showed that blocking of CR1/2 with mAb severely inhibited primary sheep red blood cell (SRBC)-specific antibody responses, whereas priming of T helper (T\(_H\)) cells (assayed indirectly as a secondary IgG-response to 4-hydroxy-3-iodo-5-nitrophenylacetyl (NIP) after boosting with NIP-SRBC) was unaffected [107].

Enhancement via IgM

Like IgE, IgG1, IgG2a, and IgG2b, IgM antibodies are able to enhance antibody response against the antigen they recognize. However, IgM is able to feedback regulate responses to suboptimal doses of particulate or large protein antigens such as malaria parasites [108], erythrocytes [1, 12, 109-111] and KLH [22, 112-114], whereas no effect on responses to small soluble proteins can be seen (unpublished observations). Enhancement by IgM is dependent on the complement system. Mice depleted of C3 by treatment with cobra venom factor have impaired antibody responses to IgM/antigen complexes, and a point-mutated monoclonal IgM, unable to activate complement, is not able to enhance the response to its antigen [115]. Monomeric IgM, which cannot activate complement, is unable to enhance antibody responses [113]. Expression of CR1/2 is required for IgM-mediated enhancement of antibody responses in mice [60]. A possible explanation to why IgM can only enhance responses to large antigens, is that when pentameric IgM binds particulate antigens, a conformational change takes place, allowing IgM to activate complement. In contrast, when IgM binds small, soluble antigens, the conformational change may not be obtained and complement cannot be activated. IgM enhances the production of specific IgM [1, 12, 14, 109, 110], IgE [116] and IgG of all subclasses [109, 117], as well as the induction of T\(_H\) cells [108] and memory cells [118].

Similarities between IgM-mediated enhancement and responses to non-complexed antigens are obvious. Both depend on an intact complement system, and both function only when suboptimal doses of antigen are used.
In fact, the observed enhancing effect of specific IgM administered together with its antigen may very well correspond to a positive feedback by natural IgM in an animal immunized with low-doses of antigen alone. Natural antibodies are antibodies mainly originating from a special subset of peritoneal B cells called B-1 cells and are present in serum without prior encounter of the antigen. Mice lacking secretory (serum) IgM but retaining serum antibodies of all other isotypes as well as surface IgM, had impaired primary antibody responses to thymus-dependent (TD) antigens and a normal response could be reconstituted by transferring IgM from naïve mice [119-121].

Early studies led to the suggestion that membrane-bound IgM (the BCR) may upon crosslinking activate complement and thus create its own ligand for the BCR coreceptor complex [88, 122]. It was subsequently shown in vitro that membrane-bound IgM cross-linked by a polyvalent ligand indeed triggers the classical complement pathway, resulting in C3 deposition on B cells [123]. Moreover, double-mutant mice lacking secretory antibodies of all isotypes as well as carrying surface IgM unable to bind C1q, could not deposit C3 on B cells upon BCR ligation, whereas single-mutant mice lacking secretory antibodies of all isotypes were able to do so [124].

Enhancement via IgG3

IgG3 antibodies can activate complement efficiently, both via the alternative pathway and, as shown using a monoclonal anti-erythrocyte IgG3, via the classical pathway [125]. IgG3 molecules are able to spontaneously self-associate (cooperative binding) [126] and to form precipitates, called cryoglobulins, in the cold [127-129]. Pathogenic cryoglobulins are found in several autoimmune diseases [130-132]. The specificity of IgG3 is mainly directed against repeating epitope antigens and carbohydrates [126, 127, 133]. Murine IgG3, as well as its human equivalent IgG2, is the predominantly responding IgG subclass against T-cell-independent type 2 antigens, whereas its production only constitutes a small fraction of the response against TD protein antigens [133, 134]. Murine IgG3 is therefore thought to be important against bacterial infections. Mice lacking IgG3 were more susceptible to pneumococcal sepsis than wt mice [135], and the ability of IgG3 to protect against Candida albicans infections relies upon its ability to activate complement [136]. It was reported in 1981 that rosette formation took place when murine macrophages were mixed with monoclonal IgG3/SRBC complexes [137]. 17 years later FcγRI was identified as a putative IgG3 receptor [138].
It was recently shown that IgG3 is able to enhance antibody responses in mice [139]. Carrier-specific antibody responses increased several hundred-fold when bovine serum albumin (BSA)-TNP or OVA-TNP was administered i.v. in complex with TNP-specific IgG3 mAbs. Enhancement by IgG3 depended on complement, since responses were severely impaired in animals depleted of C3 by CVF-treatment as well as in animals lacking CR1/2. Moreover, enhancement by IgG3 was fully functional in FcRγ-/- mice.

Figure 4. A likely mechanism behind IgM- and IgG3-mediated enhancement is co-crosslinking of CR2 and BCR by complement-containing immune complexes. Reprinted, with permission, from [2].
The present investigation

Aims
The aim of this work was to clarify the mechanisms behind antibody feedback regulation, with emphasis on CD4+ T cell responses. More specifically, the following questions have been addressed:

Paper I
- Can murine splenic B cells present IgE/antigen-complexes and activate antigen-specific splenic T cells in vitro?
- Are B cells from transgenic mice overexpressing CD23 able to enhance presentation of IgE-complexed antigen in vitro?
- How do transgenic mice overexpressing CD23 respond to IgE-complexed antigen in vivo?

Paper II
- Can IgG3 enhance antibody production in mice lacking FcγRI (CD64)?
- Are specific T cells activated by IgG3/antigen complexes in vivo and in vitro?

Paper III
- Is T cell proliferation (antigen-presentation) impaired in mice lacking CR1/2?

Experimental systems
Mice
All studies were performed in murine systems, either in vivo or in vitro. The strains used are presented in Table 1. The common denominator for the three papers of this thesis is the use of the DO11.10 mouse strain. This is a transgenic strain carrying a construct that contains rearranged TCRα and
TCRβ genes encoding a TCR specific for ovalbumin (OVA)\textsubscript{323-339} bound to I-A\textsuperscript{d} class II molecules. These animals were used as a source of OVA-specific T\textsubscript{H} cells for adoptive transfer experiments as well as \textit{in vitro} proliferation assays (see below). The DO11.10 mice were bred onto a BALB/c background for >15 generations. All strains used in these experiments were either BALB/c mice (expressing I-A\textsuperscript{d}) or genetically modified mice bred onto a BALB/c background (Table 1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Papers</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO11.10</td>
<td>Expressing an OVA-specific TRC on most T cells</td>
<td>I, II, III</td>
</tr>
<tr>
<td>CD23\textsuperscript{-/-}</td>
<td>Lacking the low-affinity receptor for IgE, CD23</td>
<td>I</td>
</tr>
<tr>
<td>CD23Tg</td>
<td>Overexpressing CD23 on B cells and T cells</td>
<td>I</td>
</tr>
<tr>
<td>CD64\textsuperscript{-/-}</td>
<td>Lacking the high-affinity receptor for IgG, Fc\gamma RI, CD64</td>
<td>II</td>
</tr>
<tr>
<td>Cr2\textsuperscript{-/-}</td>
<td>Lacking complement receptors 1 and 2, CR1/2</td>
<td>III</td>
</tr>
</tbody>
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Table 1. Genetically modified mouse strains used in this thesis.

Adoptive transfer of T-cells
Magnetically isolated CD4\textsuperscript{+} DO11.10 spleen cells were adoptively transferred i.v. into recipient mice the day before immunizations. The fate of these OVA-specific T cells could then be followed by flow cytometry or confocal microscopy by means of a clonotypic monoclonal antibody, KJ1-26, specific for the transgenic DO11.10 TCR.

Immunizations
To study antibody-mediated enhancement (paper I, II) the effect of TNP-specific antibodies of different isotypes on antibody- and T cell responses to OVA-TNP were studied \textit{in vivo}. Mice were immunized i.v. with antigen alone or with preformed antigen/antibody complexes (paper II) in physiological salt solutions. An alternative way of immunizing (paper I) was to administer antibody 30 minutes prior to immunization with antigen. By administering the antibodies in advance, it could be argued that the experimental model is more likely to resemble the \textit{in vivo} situation. However, we have seen that the effect is no different from that of preformed
antigen/antibody complexes, and both methods have been used. In some experiments mice were immunized i.v. with KLH, (paper II) or with SRBC (paper I) as a specificity control. To study the complement-dependent antibody- and T cell responses to SRBC (paper III), OVA was covalently coupled to SRBC. Mice were then immunized i.v. with OVA-SRBC, or SRBC as a control. In paper III, mice were also immunized with lipopolysaccharide- (LPS-) NIP i.p. and with Ficoll-NIP i.v. in order to study antibody responses to thymus-independent (TI) antigens.

Assays for antibody- and T cell responses
Serum Ab responses from immunized mice were quantified by enzyme-linked immunosorbent assay (ELISA). Antigen-specific levels of IgM, total IgG, or subclasses of IgG were measured by OVA-, KLH-, SRBC- or NIP-specific ELISA. Any antibodies injected in association with immunization were TNP-specific and did not interfere. The amount of adoptively transferred CD4⁺ DO11.10 cells in spleens on day 2-4 post-immunization was assayed by flow cytometry and confocal microscopy.

In vitro studies (paper I, II)
To investigate the role of antigen presentation in antibody-mediated enhancement, we performed in vitro studies using DO11.10 T cell proliferation to quantify OVA processing and presentation. This was done by culturing spleen cells with OVA-TNP with or without TNP-specific antibodies. After 18 hours, cultures were irradiated and magnetically isolated CD4⁺ DO11.10 cells were added to the cultures. Proliferation of T cells was measured as the incorporation of radioactive thymidine.

Statistical analysis
Statistical differences between groups were determined by Student’s T-test: p≥0.05 (not significant, NS); p<0.05 (*); p<0.01 (**); and p<0.001 (***).
Results and discussion

IgE enhances specific antibody and T cell responses in mice overexpressing CD23 (Paper I)

**IgE-mediated antigen presentation in vitro is dependent on CD23+ B cells**

We have used an *in vitro* antigen presentation assay where proliferation of OVA-specific T cells is assumed to reflect the amount of processed OVA presented on MHC-II. Undeniably, the cardinal feature of IgE-mediated enhancement is CD23 dependence [27, 30]. Therefore, in order to confirm that the *in vitro* system would require CD23-expression, spleen cells from wt mice and CD23−/− mice were cultured in the presence of IgE-anti-TNP and OVA-TNP and then irradiated (to prevent them from proliferating). OVA-specific T cells from the DO11.10 mouse strain were added and assayed for [3H]-thymidine incorporation. Whole spleen cells from wt mice used as APC, induced T cell proliferation at 10- to 100-fold lower concentrations of OVA-TNP in the presence of IgE-anti-TNP. However, when CD23−/− cells were used as APC, IgE-complexed OVA could not enhance proliferation of OVA-specific T cells. IgG2a-anti-TNP, used as a positive control, was able to enhance presentation of OVA-TNP on wt as well as on CD23−/− cells (Paper I, Fig 1).

Following this, we wanted to examine the role of B cells in IgE-mediated enhancement of antigen presentation *in vitro*. B cells (CD19+ or CD43−) as well as non-B-cells (CD19− or CD43+) were purified from BALB/c spleen and used as APCs *in vitro*. The ability of the CD19+ B cell population and the CD43− B cell population to present IgE-complexed antigen was comparable to whole spleen cell APCs. However, when the CD43− or the CD19− non-B-cell population was used as APC, IgE could no longer enhance antigen presentation. In contrast, IgG2a-anti-TNP, used as a positive control, was able to enhance presentation of OVA-TNP in all five APC populations (Paper I, Fig 2).

The fact that IgG2a-complexed antigen could be presented by non-B-cells (Paper I, Fig 2) was expected, since both dendritic cells and macrophages (CD43− CD19− cells) express FcγRs and MHC-II. However, the observation that B cells could present IgG2a-complexed antigen (Paper I, Fig 2) is noteworthy. In the murine system, the only FcγR expressed on the B cell is the inhibitory FcγRIIB1. It has been proposed that B cells cannot take up IgG-complexed antigen since FcγRIIB1, due to a long cytoplasmic tail, is prevented from being internalized via clathrin-coated pits [140, 141]. On the
other hand, more recent data suggests that the B cell is in fact capable of presenting IgG-complexed antigen via an FcγRIIB1-dependent pathway [142, 143].

On the mechanism behind IgE-mediated enhancement of antibody responses

It has been known for a long time that specific IgE can enhance antibody responses to small soluble antigens in vivo. In spite of this, the mechanism is not fully understood. In paper I, the problem is approached from an in vitro perspective. Since most previous in vitro studies of IgE-mediated enhancement rely on observations from EBV-transformed B cells and/or different immortalized T cell clones [36-40], we wanted to use a system that better resembled the in vivo situation. In paper I, we show for the first time that B cells, obtained directly from BALB/c spleens, are able to present IgE-complexed OVA to naïve OVA-specific T cells obtained directly from spleens of transgenic mice, and that such an in vitro system is fully functional.

IgE could enhance antigen presentation when CD19+ or CD43- cells, but not CD19- or CD43+ cells, were used as APCs (Paper I, Fig 2), confirming that the B cell is indeed the effector cell. The use of a B cell population depleted of CD43 by negative selection was a complement to the use of CD19+ B cells, ensuring that the B cells (CD43- cells) were “untouched”. IgE-mediated enhancement of antibody responses in vivo is preceded by an antigen-specific T_{H} cell expansion as well as activation, and requires CD23+ B cells [41]. These in vivo studies could not exclude that an intermediate step, mediated e.g. by dendritic cells, was needed for induction of T cells. However, the novel finding that B cells alone can present IgE-complexed antigen to naïve T cells in vitro (Paper I, Fig 2) excluded the requirement for an intermediate step, at least in vitro.

The previous in vivo observations [41], as well as data presented in paper I, are compatible with the hypothesis that IgE-mediated enhancement occurs via enhanced antigen presentation on B cells, resulting in better T cell help, which in turn induces better antibody responses. The physiological role of IgE-mediated enhancement is not clear. Serum levels of specific IgE-antibodies are extremely low, and present only after class switching. Therefore, it can be argued that the high levels of specific IgE given to mice during immunization, do not represent a normal biological situation, and that these effects seen by IgE-mediated enhancement would not be clinically representative. Nevertheless, following a secondary immune response, higher production of specific IgE may occur locally. In addition to this, CD23 on B cells may already be pre-loaded with IgE in these specific locations, waiting for antigen to bind. In view of this, it is possible that IgE-
mediated enhancement is important in e.g. the pathology of allergies. In fact, in an autologous system with T- and B cells derived from the same atopic dermatitis patient, serum containing IgE-anti-Der p II (major house dust mite) enhanced antigen presentation on EBV-transformed B cells to T\textsubscript{H} cells in vitro [39]

**B cells overexpressing CD23 function as APCs for IgE-complexed antigen in vitro**

CD23Tg mice [54] displayed a suppressed antibody response when immunized with antigen in alum [54, 55]. We wanted to test whether B cells from CD23Tg mice were able to present IgE-complexed antigen. In our *in vitro* system, CD19\textsuperscript{+} spleen cells from CD23Tg mice enhance presentation of IgE-complexed antigen equally well as CD19\textsuperscript{+} wt spleen cells (Paper I, Fig 3). This implies that although CD23Tg mice show a suppressed antibody response when immunized with antigen in alum, B cells from these mice are normal concerning IgE-mediated enhancement in vitro.

**IgE enhances T cell responses as well as antibody responses in CD23Tg animals in vivo**

We next compared T cell- and antibody responses to IgE-complexed antigen in CD23Tg and wt mice. Mice were adoptively transferred with CD4\textsuperscript{+} DO11.10 T cells and immunized i.v. with OVA-TNP alone, IgE-anti-TNP + OVA-TNP or with IgE-anti-TNP alone. The percentage of OVA-specific T cells in the spleen was significantly enhanced by IgE in both wt and CD23Tg mice (Paper I, Fig 4). Comparable stimulation indices as well as percentages of KJ1-26\textsuperscript{+} CD4\textsuperscript{+} cells were seen in both groups. To visualize the T cell expansion, spleen sections were stained for confocal microscopy (Paper I, Fig. 5). A large amount of proliferating OVA-specific T cells were seen in both wt and CD23Tg mice following immunization with IgE/OVA-TNP. In contrast, only a relatively small number of T cells could be traced in animals immunized with OVA-TNP alone or with IgE-anti-TNP alone. Antibody responses in wt and CD23Tg mice immunized with OVA-TNP alone or with IgE-complexed OVA-TNP were analyzed. IgE enhanced the IgG-anti-OVA responses on day 7-28 post-immunization in both strains and similar stimulation indices were seen (Paper I, Fig. 4).

Interestingly, a general reduction of antibody responses was seen in CD23Tg animals to OVA-TNP as well as to IgE/OVA-TNP (Paper I, Fig. 4). Since IgG-responses to both SRBC alone and KLH alone were lower in CD23Tg animals (Paper I, Fig. 6) it is unlikely that the effect was due to IgE/CD23-interactions. One possible explanation is that the transgene in CD23Tg-mice by an unknown mechanism lead to a defective antibody production.
IgG3-mediated enhancement of the antibody response is normal in FcγRI-deficient mice. (Paper II)

We previously showed that IgG3-mediated enhancement is complement-dependent since responses were severely impaired in complement-depleted animals as well as in animals lacking CR1/2 [139]. Enhancement by IgG3 appeared to be independent of Fc-receptors, as it was fully functional in FcRγ−/− mice. However, since IgG3 has been shown to bind FcγRI [138, 139], and since FcRγ−/− mice still express 20% of this receptor [144], Fc-dependence could not be completely ruled out. In the present study we have used mice specifically lacking FcγRI to see whether this receptor is involved in IgG3-mediated enhancement.

No role of FcγRI in IgG3-mediated antibody responses in vivo
The importance of FcγRI in IgG3-mediated enhancement was investigated by immunization of wt and FcγRI−/− mice with antigen or IgG3-complexed antigen. Serum titers of antigen-specific IgG were followed for three weeks, and were similarly enhanced by IgG3 in wt and FcγRI−/− mice (Paper II, Fig 1). Control mice immunized with IgG3 alone had no serum titers of antigen-specific antibodies, confirming that there is no unspecific effect of IgG3. In addition, no animals had enhanced titers of antibodies against KLH, an antigen not recognized by IgG3, illustrating the specificity of IgG3-mediated enhancement.

Minor effect on T cell responses by IgG3 in vivo
The role of T cells in IgG3-mediated enhancement was investigated by adoptive transfer of antigen-specific DO11.10 T cells to wt or FcγRI−/− mice, followed by immunization with antigen alone, IgG3-complexed antigen or (as positive controls) IgE- or IgG2a-complexed antigen. The ability of IgG3 to enhance T cell proliferation was small in both strains: the number of antigen-specific T cells (CD4+, KJ1-26+) in spleens three days after immunization was increased by 1.4- to 3.2-fold by IgG3 (Paper II, Table 1 and Fig 2). In contrast, a 6- to 11-fold expansion was seen by IgE and a 4.2- to 4.5-fold expansion by IgG2a.

No effect on T cell responses by IgG3 in vitro
The ability of IgG3 to induce T cell responses was further assayed in vitro. Spleen cells from wt mice were incubated with either antigen alone or antigen together with specific IgG3 or (as a positive control) specific IgG2a. IgG3-complexed antigen could not induce T cell proliferation, whereas IgG2a-complexed antigen significantly enhanced T cell proliferation (Paper II, Fig 3).
On the mechanism behind IgG3-mediated enhancement of antibody responses

The antibody responses to IgG3-complexed antigen are similar in wt mice and in mice lacking FcγRI (Paper II, Fig 1), confirming the idea that IgG3-mediated enhancement mainly operates via complement, and that the role of Fc-receptors is insignificant. The possibility that Fc receptors act redundantly to the complement system is unlikely, since our previous report shows an almost complete abolishment of IgG3-mediated antibody responses in CR1/2-deficient animals having normal FcγRs [139, 145].

We also for the first time analyzed the ability of IgG3-complexed antigen to induce proliferation of antigen-specific T cells. TNP-specific IgE and IgG2a, administered together with OVA-TNP, enhance OVA-specific CD4⁺ T cell responses in vivo, most likely owing to increased antigen presentation [41, 67]. In the present study we show that IgG3, compared to IgE and IgG2a, is a poor enhancer of T cell proliferation in vivo and has no effect on T cells at all in vitro. The minor effect on T cells seen in the in vivo experiments may be a fortuitous finding, or simply a result of increased IgG3-induced aggregation of antigen. Nevertheless, it cannot be ruled out that IgG3 indeed has a minor effect on antigen presentation, since IgG3-complexed OVA enhanced proliferation of OVA-specific T-cells 1.7- to 2.8-fold in wt mice (Paper II, Table 1, left column). Importantly, this was not an effect of antigen capture via FcγRI, as T-cell proliferation in FcγRI⁻ mice immunized with IgG3-complexed OVA was slightly higher than in corresponding wt mice (Paper II, Table 1, experiment 1).

The minor effect of IgG3 on T cell responses suggests that antigen presentation is not a major mechanism of IgG3-mediated enhancement. This leaves us with two possible hypotheses of how IgG3 can enhance antibody responses to small soluble antigens. First, IgG3 may form immune complexes that contain complement, possibly via activation of the classical complement pathway and aided by its ability to self-aggregate. These immune complexes may then be captured by CR2 on FDCs, leading to increased exposure of antigen to specific B cells. The second, not mutually exclusive, hypothesis is that the complement-containing immune complexes co-crosslink CR2 and the BCR on a specific B cell, lowering the threshold for B cell activation, as shown to take place in vitro [92, 95, 96].
Impaired antibody responses but normal proliferation of CD4⁺ T cells in mice lacking complement receptors 1 and 2. (Paper III)

The mechanism(s) behind impaired antibody responses to large or particulate TD antigens, such as erythrocytes, in mice lacking Cr2 is not fully understood. Since antigen presentation via uptake by CR2 on B cells has been implied as a possible mechanism in vitro [98-104], we have tested whether presentation to T cells also plays an important role in vivo. We made use of the DO11.10 adoptive transfer system (enabling the detection of OVA-specific CD4⁺ T cells) to compare T cell responses and antibody response to OVA-SRBC in Cr2⁻/⁻ and wt mice.

**Cr2⁻/⁻ mice have impaired IgG-anti-SRBC-responses over a wide range of antigen doses.**

Previous studies have shown that low doses of large or particulate antigens administered i.v. require CR1/2 for a normal antibody response to take place. When higher doses of antigen are used there is no need for complement [88]. Because of this, we first needed to establish at what doses the antibody response to OVA-SRBC required CR1/2-expression. Cr2⁻/⁻ and wt mice were immunized with 5×10⁶ to 5×10⁸ OVA-SRBC/mouse. Antibody responses were severely impaired in the Cr2⁻/⁻ groups regardless of whether the animals had been given high or low doses of antigen (Paper III, Fig 1A-E).

**Impaired antibody responses to OVA-SRBC but normal proliferation of OVA-specific CD4⁺ T cells in Cr2⁻/⁻ mice.**

We next wanted to see if induction of CD4⁺ T cells was impaired in Cr2⁻/⁻ mice where antibody responses to OVA were impaired. Wt and Cr2⁻/⁻ mice were adoptively transferred with OVA-specific CD4⁺ DO11.10 T cells and subsequently immunized with 1×10⁸ or 5×10⁷ OVA-SRBC, or with unconjugated SRBC as a control. The number of OVA-specific T cells in spleens at day 2-4 was determined, and serum levels of IgG-anti-SRBC and IgG-anti-OVA were followed. Although antibody levels in Cr2⁻/⁻ mice were impaired, no difference in Th1 cell induction could be detected (Paper III, Fig 2). In other words, the severe impairment of antibody responses is not reflected in any impairment of CD4⁺ T cell responses in the absence of CR1/2.

**Impaired antibody responses to TI antigens in Cr2⁻/⁻ mice.**

The requirement for complement in normal antibody responses to suboptimal doses of TI antigens has previously been demonstrated in vivo [146, 147]. To compare the antibody responses to TI-antigen in Cr2⁻/⁻ and wt mice, different doses of LPS-NIP were administered i.p. and different doses of Ficoll-NIP were administered i.v. Poor IgG-anti-NIP responses to both
antigens were seen in Cr2−/− mice (Paper III, fig 3). Since these antigens are TI, they do not require presentation to Th cells.

On the mechanisms controlling impaired antibody responses in Cr2−/− mice

Many in vitro studies have demonstrated that B cells can endocytose C-containing immune complexes via CR2, and present antigen to Th cells [98-100, 102-104, 148]. The molecular mechanism(s) behind why normal antibody responses in vivo require the classical pathway are not completely understood. Our main purpose of this study was to determine whether lack of appropriate presentation to CD4+ T cells of antigen endocytosed via CR2, could explain the extremely low antibody responses observed by many authors in Cr2−/− animals [83-87]. If defective antigen presentation is a major mechanism behind lower antibody responses in mice lacking CR1/2, lower T cell activation as well as antibody responses would be expected. Our finding, that T cell proliferation is normal in spite of impaired antibody responses in Cr2−/− mice, point to a minor role of antigen presentation. These findings support those of a previous in vivo study, where CR1/2 were blocked by mAbs, and priming of Th cells, indirectly assessed in a hapten-carrier system, was normal in spite of severely inhibited antibody responses [107]. Our findings also agree with a study of the role of C3 and C4 in T cell priming [78].

Our second observation is that antibody responses to TI-1 and TI-2 antigens are impaired in Cr2−/− mice. Since these antigens do not require processing and presentation to Th cells, the observation that they induce poor responses in Cr2−/− mice argues against that antigen presentation is the only mechanism behind the impaired antibody responses in Cr2−/− mice. It has been previously shown, in studies where C3 was depleted by CFV and where CR1/2 was blocked by mAbs, that the complement system plays a role also in antibody responses to TI antigens [146, 147, 149]. Thus, although studies undoubtedly show that CR2 is able to mediate endocytosis of C-tagged immune complexes in vitro [98-100, 102-104, 148], the findings by us and others do not support a role of this mechanism in vivo. Admittedly, it cannot be excluded that antigen presentation operates also in vivo, but at present other mechanisms such as increased B cell signaling [92, 103] and/or increased capture of C-containing immune complexes by FDC [94, 150] appear more likely.
Conclusions

Paper I
- The mechanism behind IgE-mediated enhancement in vitro is increased antigen presentation on B cells via CD23.
- B cells from transgenic mice overexpressing CD23 can present IgE-complexed antigen in vitro and in vivo.
- Antibody responses to a variety of TD antigens are lower in mice overexpressing CD23.

Paper II
- IgG3-mediated enhancement is normal in the absence of FcγRI.
- Enhanced antigen presentation to TH cells is not a major mechanism of IgG3-mediated enhancement.

Paper III
- Antigen-uptake and presentation on B cells is not the primary mechanism behind the requirement of CR1/2 for antibody responses to SRBC.
Acknowledgements

This work was performed at the Department of Genetics and Pathology, Uppsala University. I wish to acknowledge all the people with whom I have been in contact during these years. Without a doubt you have all in various ways contributed to the finishing of this thesis. In particular I wish to express my sincere gratitude and special word of thanks to the following persons for their help, guidance and encouragement

Birgitta Heyman, my supervisor and dear friend. Thank you for sharing your vast immunological knowledge, for being an outstanding mentor, for ALWAYS being able to spare a moment to discuss findings or plan new bold experiments, for keeping me enthusiastic in times of despair and for your ability to see a positive side of a negative result.

Imma Brogren, our excellent lab technician, for all the help, support and for providing the lab with a merry atmosphere. Without you, things would no doubt have been an utter chaos.

Andy Getahun. Truly one of the smartest, wittiest and most generous individuals I ever have had the fortune to meet. Without your help this would not have been possible. I am sure you will become a very famous scientist one day. Good luck with everything you do in life.

Fredrik Hjelm. Lillegull. Nemesis. Brother-in-arms. Thank you for all the help in- and outside the lab, for all the absurd practical jokes, relaxing fishing excursions, and for being a great friend.

My co-supervisor Sandra Kleinau for good advices, suggestions and discussions.

Past and present members of the Heyman/Kleinau groups: Kjell-Olov Grönvik for good advices, suggestions and questions. Ingrid Dahlbom for teaching me about coeliac disease. Anna Bergman for sharing good karma during critical experiments. Christian Rutemark for being such an annoyingly cheerful bugger all the time. Annika Hermansson for saving my butt during the last couple of months. Micke, Sara, Jörgen, Ravi, Kicki, Joey, Oskar and Kristina. A very special thanks to Maria Andrén, Sofia Magnusson, Kajsa Nilsson and Ida Waern for countless laughs and interesting work-related and work-unrelated discussions.

Jan Grawé for flow cytometry assistance

Daniel Färnstrand for linguistic improvements of the thesis.
My examiner Kenneth Nilsson
All the, many times overlooked, people that make sure everything is working properly: administrators, animal technicians and cleaning staff. Without you guys there would be no time left for research. Vahik for his delicious lasagnas.

Wing-Shing Cheng, my oldest, dearest friend from back in the days. Discussions on life as well as science over numerous Siedler-sessions are gratefully remembered. It doesn’t at all feel like ten years, does it? Oh, and I never lost the bike btw ;-)  

Björn Carlsson for tips regarding flow cytometry, for your never-ending supply of hilarious anecdotes and movie recitations, as well as for never refusing a pint of Old Speckled.

Helena Dzojic. For constantly wearing a contagious smile and for helping out with reagents and equipment in times of emergency. You carry the biggest heart I know, and I am so proud of being your friend. I wish you success, happiness and fortune with everything you do.

The rest of the staff at Clinical Immunology, for lunch dating and for making me feel like a worthy citizen of the Klinimm-realm in spite of my residing on the third floor. Particularly Sofia, Ole, Alo.

All past and present colleagues residing in our writing room: Micke J, Mats, Maria E, Pernilla, Fredrik J, Umash et al, + the friendly people on the third floors.

Lina, Emil, Rolf, Tanya, Tossa and Malin for mature intellectual discussions during late hours. Björn Åkerblom for sharing interests in boardgaming, frisbeeing and homebrewing. Linus and Bertil for occasional visits and for keeping in touch in spite of long distances.

Lena, Benny, Patrick, Heléne and Andreas for Regal dinners, forest excursions, and for teaching me how to brew beer. For showing me Hälsingland. For your understanding, help and support in good times as well as in bad.

Mom. For everything. I love you so much.

Janne, Marie, Amanda and Jacob for much needed time off in Göteborg when in fact there was no spare time. Ann-Marie, Christer, Tina and Markus for being the coolest people I know. You are all an inspiration to me. Dad and Anna-Lena for always being there when things are tough. For encouragement, for love, for everything.

Therése, I cannot think of anything you haven’t done to help and support me during these years. Words do not begin to describe what you mean to me, and I’m so happy you are mine. I love you.

This work was financially supported by the Agnes & Mac Rudberg’s Foundation; Ellen, Walter and Lennart Hesselman’s Foundation; Hans von Kantzow’s Foundation; King Gustaf V:s 80 Years foundation; Lily and Ragnar Åkerham’s Foundation; Ankarstrands Foundation; The Swedish Research Council; Ollie and Elof Ericsson’s Foundation and Uppsala University.
References


60. Applequist, S.E., et al., Antibody production in mice deficient for complement receptors 1 and 2 can be induced by IgG/Ag and IgE/Ag, but not IgM/Ag complexes. J. Immunol., 2000. 165: p. 2398-2403.


Acta Universitatis Upsaliensis

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