Centre for Immune Regulation

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Anti-Idiotypic B cells and Idiotype-specific Th cells in the context of Id^+ Ig: interaction and mechanisms of regulation.

Doctoral thesis by Johanne T. Jacobsen

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Oslo, November 2011

Johanne Tracey Jacobsen

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ABBREVIATIONS

aa	Amino acid
Ab	Antibody
ADCC	Antibody dependent cell cytotoxicity
AICD	Activation induced cell death
anti-Id^{DKI}	Anti-Id double knock-in
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
CDR	Complementary determining region
CFA	Complete Freund's adjuvant
DC	Dendritic cell
DNP	2,4 Dinitrophenol
ER	Endoplasmic reticulum
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcR	Fc-receptor
FcRn	Neonatal FcR
FDC	Follicular dendritic cell
HC	Heavy chain
HEL	Hen egg lysozyme
HEV	High endothelial venules
Id	Idiotope
Ig	Immunoglobulin
Ii	Invariant chain molecule
ITAM	Immunoreceptor tyrosine-based activation motif
KI	Knock-in
LC	Light chain
LN	Lymph node
LPS	Lipopolysaccaride
MHC	Major histocompatibility complex
mIgD	membrane IgD
MZ B cell	marginal zone B cell
NIP	4-hydroxy-3-iodo-5-nitrophenylacetic acid
NK cell	Natural killer cell
RSS	Recombination signal sequence
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TF	Transcription factor
Th	T follicular helper
TNP	2,4,6-trinitrophenyl
T_{reg}	Regulatory T cell
VDJ	variable, diversity, joining

LIST OF PUBLICATIONS

Paper 1

The cellular mechanism by which complementary Id^+ and anti-Id antibodies communicate: T cells integrated into idiotypic regulation

Johanne T. Jacobsen, Elin Lunde, Vibeke Sundvold-Gjerstad, Ludvig A. Munthe and Bjarne Bogen.

Immunology and Cell Biology (2010) **88,** 515–522

Paper 2

B lymphoma cells with mutually binding B cell receptors kill each other: a mechanism for reduced idiotypic connectivity?

Johanne T. Jacobsen, Vibeke Sundvold-Gjerstad, Frode M. Skjeldal, Oddmund Bakke, Anne Spurkland and Bjarne Bogen.

Submitted to International Immunology.

Paper 3

Anti-Id B cells and Id-specific $CD4^+$ T cells collaborate efficiently under physiological conditions.

Johanne T. Jacobsen, Karoline Schjetne, Ludvig A. Munthe and Bjarne Bogen.

Manuscript.

INTRODUCTION

Antigen presenting cells

The immune system harbors three sets of professional antigen –presenting cells (APCs): dendritic cells, macrophages and B cells (and certain activated epithelial cells). Common for the three subgroups is that they constitutively express Major Histocompatibility Complex (MHC) molecule (1). The classical MHC class I and MHC class II molecules are polymorphic membrane bound glycoproteins that require peptide to maintain a stable conformation (2). Thereby the professional APCs can at all times present antigen. Other cells can also present antigen, as most nucleated cells express MHC class I. Other cells can express low levels of MHC class II or be activated to express MHC class II. They include fibroblasts, thymic epithelial cells, thyroid epithelial cells, glial cells, pancreatic beta cells and vascular endothelial cells (1).

Foreign exogenous antigen will be endocytosed and processed by the APC, and displayed on MHC class II molecules on the cell surface. MHC class II will present peptide to $CD4^+$ T helper, which become activated and can initiate immune responses. Another antigen-presentation pathway, the MHC class I dependent pathway, presents peptides from nuclear or cytosolic proteins to $CD8^+$ T cells (1). The phenomena of "cross presentation" leads to the presentation of extracellular antigens on MHC class I molecules to $CD8^+$ T cells (3).

Dendritic cells

Dendritic cells (DC) are classically viewed as the most efficient APCs: DCs express high levels of MHC class I and II (4,5), DCs are active APCs long after the initial pulsing with antigen (6), and only few DCs are required to activate a large number of T cells (7). Also DCs constantly sample the micro environment by extending and retracting dendrites (8). There is a broad tissue localization of DCs, being distributed in non-lymfoid tissue either in tissues in contact with the external environment or interstital zones of heart, kidney and gut. Following activation, DCs migrate to lymphoid organs (1).

B cells

B cells are classically not regarded as very efficient APCs: there is a low frequency of naïve B cells with specific receptor for any particular antigen. B cells can internalize antigen by: phagocytosis, fluid phase pinocytosis and receptor mediated endocytosis. B cell receptor (BCR) mediated endocytosis, can be a highly efficient process. Batista and Neuberger demonstrated that BCR/antigen affinity is directly proportional to the B cell ability to present antigen, and at high affinity BCR/antigen interactions, minor antigen concentrations are sufficient for uptake, presentation and Th cell activation (9). (see also the General discussion).

B cell development

B cells are generated from pluripotent hematopoietic stem cells during fetal development and in the bone marrow after birth. The bone marrow will hold B cells from early progenitors to mature B cells. A common lymphoid precursor gives rise to T-, B- and Natural Killer (NK) cells. As B cells mature in the bone marrow and eventually reach peripheral locations, they express a varying pattern of surface markers (see Table 1). Several nomenclature schemes have been proposed for developmental B cell stages (10,11).

B cell development is dependent on the stromal cells for providing adhesion and soluble factors. B cell development is marked by successive steps in the rearrangement of variable (V), diversity (D), joining (J) and constant gene segments of the B cell receptor (see Fig.1) (1). The order of rearrangement according to the ordered model (Fig.1) is that heavy chains recombine first, followed by pairing with a surrogate light chain and thereafter light chain rearrangement (12-14). However evidence that this is not such a stingent process but that rearrangements occur independently is given in a stochastic model, for human (15) and mouse (16). As for kappa-, κ, and lambda-, λ, light chain expression, it is believed that there is sequential rearrangement and expression with a preference for κ (17). In mouse 95% of peripheral Immunoglobulin (Ig) is κ (18). Also in bone marrow κ light chain expression outnumbers λ light chain expression (19).

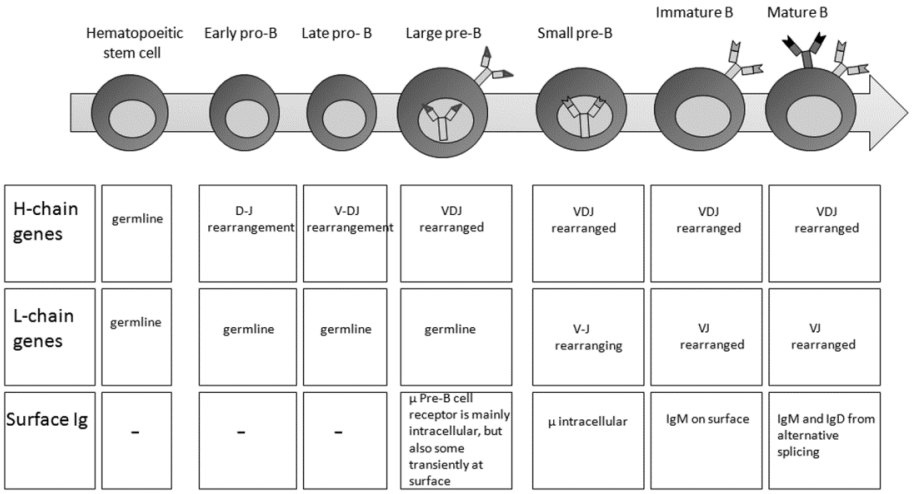


Figure 1. B cell lineage developmental stages are characterized by rearrangement and expression of Ig genes. Stem cell Ig genes are still in germline configuration (see Fig.3). Heavy chain gene segments arrange first in the order D to J, V to DJ. VDJ is assembled in late-pro B cells. A successful VDJ join, leads to expression of the μ chain, which is expressed mainly intracellularly at the Large pre-B ell stage with a surrogate light chain. Subsequently, light chain genes are rearranged at the small pre-B cell stage. When a light chain is assembled, the immature B cell will express the surface IgM molecule. Mature B cells produce both IgM and IgD from alternative splicing of mRNA (1). Figure adapted from (1).

Once the BCR is assembled, immature B cells can undergo selection for self-tolerance. The antigen specificity of a B cell will be determined early in the differentiation, when variable regions assemble from gene segments. When assembled, the affinity and specificity of the BCR is tested by binding to antigens in the immediate environment. Receptors that bind weakly with self antigen are positively selected, and receptors that bind strongly are negatively selected, leading to cell death (1). Hence a certain level of tolerance is established to self antigens. B cell tolerance is recognized as being less stringent than T cell tolerance, and it has become clear that peripheral immature B cells often possess autoreactive BCR (20). Surviving emigrant B cells become mature naïve B cells in the periphery, circulating between blood and lymphoid tissue, where they again can encounter antigen and be clonally selected in adaptive immune responses (see further details under "T cell – B cell collaboration").

B cell subsets in the periphery

Before differentiating into mature B cells, the B cells go through several transitional stages. Transitional (T) B cells comprise three subsets (21), all characterized by the marker CD93/AA4.1. There are five mature B cell subsets in mouse spleen (22). The bone marrow derived follicular B cells and marginal zone (MZ) B cells (together the B2 population), which constitute the majority of splenic B cells. In addition come B-1a and B-1b cells, which are minor subsets in spleen as well as the newly characterized regulatory B cell that shares some characteristics of the B1-a cells and MZ cells (23-25). These different subsets vary in terms of their location, ability to migrate, and in the likelihood that they will be activated in a T-dependent or a T-independent fashion (all except the newly characterized regulatory B cell reviewed in (26,27)). The surface marker profile and frequency in spleen for these five subsets is given in Table 1. MZ and B-1 cells have been extensively characterized.

Marginal zone B cells

In rodents, MZ B cells are located, mainly around the marginal sinus of the spleen, a localization promoted by Sphingosine 1-phosphate receptor (28). MZ B cells are involved in antigen transport from the marginal zone into the splenic follicles, a function that has been linked to their high CD21 expression (29). MZ B cells can participate in T independent antigen responses (30). Reports show that MZ B cells are more prone to T independent activation and generate effector cells more readily than the follicular B cells (31). MZ B cells are also involved in T dependent antigen responses both by transporting antigen to follicular B cells, but also directly by presenting antigen to Th cells (32). Several experiments suggest that the MZ compartment is more permissive to self reactive B cells than the follicular compartment (33-35).

B-1 cells

The term “B-1 cells” was used to describe a subset of peripheral B cells developing earlier than follicular B cells in B cell ontogeny (36). The B-1 cell population includes B-1a cells, which express CD5, and the B-1b cells, which are CD5 negative (37-40). B-1a and B-1b cells both reside mainly in the peritoneal and pleural cavities but have different functions. B-1a cells contribute to innate-like immunity and B-1b cells contribute to adaptive immunity (41,42). Similar to MZ B cells, B-1 cells constitute a population of cells

that are enriched for self-reactive B cell receptors (43). B-1 cells, as MZ B cells, readily generate effector cells in early stages of an immune response (44).

Table 1

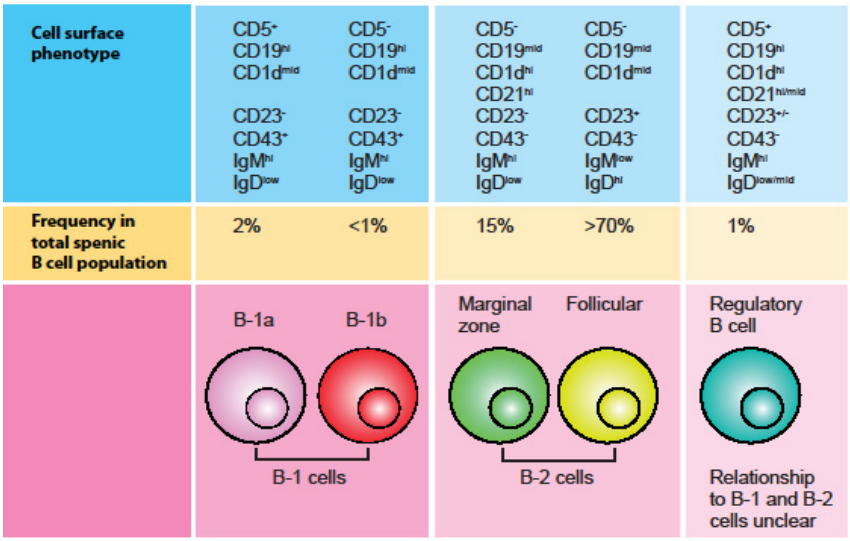


Table 1 adapted from (22)

The B cell receptor

The two last exons of each constant gene segment in the HC Ig locus, contain secreted and membrane bound form respectively. If the membrane exon is not spliced off, the Ig exists in the membrane bound form of the BCR. The BCR consists of an antigen-binding Ig with no direct signaling ability. The accompanying heterodimer Igα (CD79a) and Igβ (CD79b), contains the cytoplasmic activation motif, "immunoreceptor tyrosine-based activation motif" (ITAM), capable of signaling (1). Upon antigen binding to BCR, the tyrosines in ITAM sequences are phosphorylated. This is done primarily by the kinase LYN. Subsequently, SYK is recruited to the phosphorylated Igα-Igβ heterodimer, with the triggering of at least four different signaling cascades (45).

Antigen binding to BCR, arrests the free diffusion of the BCR monomers in the plasma membrane and triggers BCR organization into signaling clusters (46,47). Antigen induced BCR clustering enables phosphorylation of ITAMs and thereby triggers signaling. The efficiency of this process is limiting for the degree of B cell activation (48).

It has been demonstrated that isotype switched IgG enhances BCR oligomerization and thereby signaling due to the 12 membrane proximal residues of the H chain tail (49).

In addition to increased affinity due to somatic hypermutation, this mechanism may explain why IgG isotype switched B cells are more efficient than non-switched B cells *in vivo* (50,51).

Antigen processing, the endocytic pathway

APCs internalize extracellular material by several different mechanisms collectively termed endocytosis. One of the best characterized endocytic mechanisms is receptor mediated endocytosis via clathrin coated pits (52).

Clathrin dependent Receptor mediated endocytosis¨

Receptor mediated uptake by clathrin coated pits is a pathway used for processing ligand/receptor complexes. The uptake is initiated by internalization motifs in the endocytic receptor cytoplasmic tail (53-55). Adaptor proteins mediate formation of the pits (56). Clathrins organize into basket-like structures on the cytoplasmic side of membrane and form vesicles. A signal transduction pathway including the Vav1 and/or Vav3 protein isoforms and the GTPase dynamin has been shown to mediate BCR/ligand internalization (57). There are a large number of endocytic receptors on DCs: FcγRs (58,59), heat shock protein receptors (60,61), scavenger receptor (CD36) (62), mannose receptor (63).

Non clathrin mediated endocytosis

"Phagocytosis" is the cellular uptake of large particles mediated by cell surface receptors and actin (64). Receptor binding may occur directly, or indirectly by opsonization, (coating of the particle with Ig or complement and subsequent receptor binding). The "zipper" model is a widely accepted description of the mechanism of phagocytosis (65). This involves engulfment of particles via extended membrane pseudopods, with a subsequent recruitment of surface receptors interacting with opsonins or proteins on the engulfed particles. After engulfing antigen, the two pseudopods fuse and budd off into an early endosome (66).

Another type of non-clathrin mediated endocytosis is macropinocytosis. This closely resembles phagocytosis. However macropinocytosis has not been reported to concentrate receptors (67).

Antigen processing and loading onto MHC class II molecules, BCR mediated uptake as an example

BCR mediated uptake allows antigen to be presented on MHC class II molecules. The first step in the processing is internalization of the BCR antigen complex (68). The antigen is then proteolytically processed and can subsequently be loaded onto MHC class II molecules (69). Antigen degradation in the endocytic pathway occurs in a gradually more acidic and reducing environment in the endosomal/lysosomal vesicles. Endosomal acidity is maintained by ATP dependent protein pumps (70). Several proteases are required for generation of antigenic peptides (71,72).

The MHC class II molecule is synthesized in the endoplasmic reticulum (ER), whereupon it is complexed to the Invariant chain molecule (Ii) in a specific conformation (73). This binding prevents the binding of ER-peptides prematurely (74). Ii accompanies the MHC class II molecule through the golgi complex to the endosomal compartments (1). In a particular subpopulation of the endosomal compartments the MHC class II associated invariant chain is removed by the action of proteases and the protein HLA-DM/H-2M (75) (human version). The resulting peptide loaded MHC class II can continue to cell surface. In mouse B cells, antigen processed via the transferrin receptor is presented 10-100 times less efficiently than same antigen processed via the BCR (76). This feature is possibly due to the transferrin receptors limited access to intracellular MHC class II compartments (77).

Traditionally, distinction has been made between MHC class II molecules on APCs ,exclusively presenting exogenous antigens, and MHC class I molecules presenting proteasome degraded “self” cellular components or intracellular pathogens that are loaded onto MHC class I in the ER. Hence the classical pathways for presentation of exogenous and endogenous antigen; have been termed the endogenous (MHC class I associated) and exogenous (MHC class II associated) pathways, respectively (1). However, more recent reports show that endogenously synthesized antigens are also presented on MHC class II molecules (78-80). Peptides eluted from MHC class II molecules are in fact mainly secretory pathway peptides produced from endogenously produced proteins (81-83).

Immunoglobulins

By dissociation of the Ig monomer by reducing disulfide bridges, it was determined that Immunoglobulins (Igs) consist of two identical heavy chains (~50 kDa) and two

identical light chains (~25 kDa) (H_2L_2) (84). The antigen binding site of Ig is composed of three loops within the H and L chain, called the hypervariable regions 1-3 or complementary determining regions 1-3 (CDR1-3). The variable and constant regions (see Fig.2) of the Ig can be classified into the following structural entities; Fragment Antigen Binding, Fab and Fragment crystallizable, Fc, respectively (85). Ig structure is given in Fig.2.

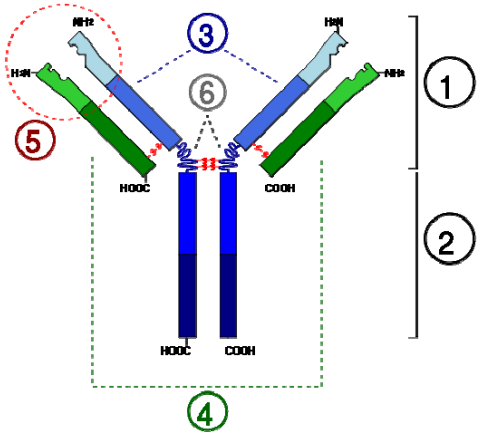


Figure 2. Ig structure. Fab fragments [1] are joined to the Fc region [2] by the hinge region [6]. Each HC [3] holds one amino terminal (NH_2) variable region (light blue) and three carboxy terminal (COOH) constant regions (darker blue). Each LC [4] also holds an NH_2 terminal variable region (light green) and one COOH terminal constant region (dark green). The two identical HCs are bound to each other covalently by disulfide bridges (-S-S-). The V regions of the HC and LC combined constitute the antigen specificity of the Ig [5, stipled red circle]. The figure is copied from http://en.wikipedia.org/wiki/Antibody

The constant regions of the Heavy chain (HC) and light chain (LC) (Fig.2) can be of different isotypes: for LC κ or λ and for HC, in mammals; IgG, IgM, IgA, IgD and IgE and in addition the subclasses IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2 in humans (1). The HC isotypes will be further discussed under "Immunoglobulin functions".

Immunoglobulin diversity

As shown in Fig.2, the paired variable domains together constitute the antigen specificity of the Ig. The term idiotope (Id) is used for the variable region antigenic epitope of an individual Ig. The total Id repertoire in humans has been estimated to 10^{11} (1). This multitude of Ids is achieved by several processes i) combinatorial variation, due to combination of different gene segments in the Ig HC and LC locus (86), ii) junctional diversity, as a result of the template independent addition or deletion of nucleotides at the joints of the joined segments (1,87), iii) combinatorial diversity as a function of possible HC and LC chain V region pairings (1) and iv) the process of somatic hypermutation that introduces point mutations in rearranged V genes, secondary to antigen dependent GC

formation (88). The assembly of antigen receptor genes is mediated by a V(D)J recombinase consisting of RAG-1 and RAG-2 proteins (89,90). This RAG complex targets recombination signal sequences (RSSs) flanking all Ig gene segments (91). The V(D)J recombination process is performed stepwise as shown in Fig.1.

The immunoglobulin locus in mice (and humans)

The murine Ig HC locus is ~3 million bases in size and is located on chromosome 12. The murine Ig LC locus is located on chromosome 6 and 16 for κ and λ respectively (92). Within the genus Mus musculus, the Ig loci are highly polymorphic (93). The Ig HC loci of inbred strains have been assigned to different haplotypes (94). The BALB/c strain used for generation of knock-in (KI) mice herein has the Ig HC a haplotype (95). A comparison of the murine and the human Ig locus (HC + LC) is given in Fig.3. As far as possible IgH and IgL chain V (D) and J gene segments are given for BALB/c (only partially annotated as of today).

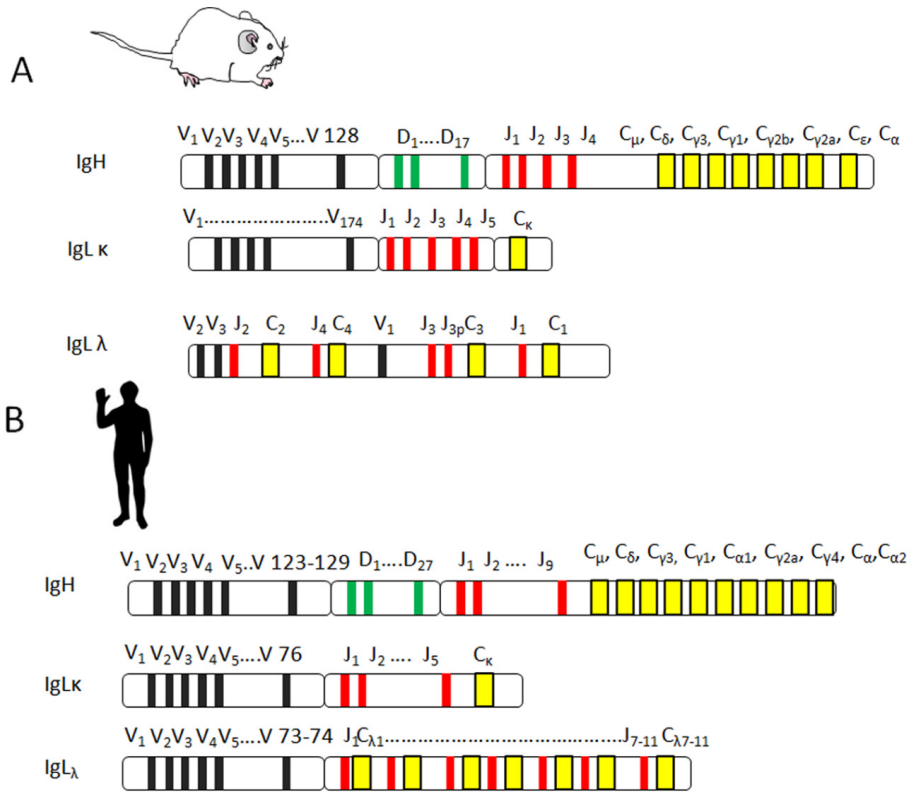


Figure 3. The murine and human HC and LC Ig locus. A. Murine IgH and IgL locus organization. Ig HC V genes:128 V genes, whereof 49 are functional (96). Ig HC D genes: 17 genes where of 14 are functional (92,97). Ig HC J genes: 4 functional genes (98). Constant region: 8-9 functional genes, only 8 shown (92). For murine κ LC locus: 174 V genes have been annotated, whereof 94-96 are functional, 5 J genes, whereof 4 are functinal and 1 functional constant gene (92). For Ig LC λ locus there are 3 functional V genes arranged in two clusters, 5 J genes, whereof 3 are functional (p = pseudo gene), and 4 constant genes whereof 2-3 are functional (92,99). B. Human Ig HC and Ig LC locus organization. HC V genes: 123-129 genes, whereof 38-46 are functional. HC D genes: 27, whereof 23 are functional. HC J genes: 9 genes whereof 6 are functional. HC constant genes: 9 functional genes (of 11 genes, only functional genes are shown). For human κ LC locus there are 76 V genes whereof 31-35 are functional, 5 functional J genes and one functional constant gene. For human λ locus there are 73-74 V genes whereof 29-33 are functional, 7-11 J and constant genes in clusters, whereof 4-5 (J-constant) clusters are functional (92). Color codes: V = black, D = green, J = red, constant = yellow. Dots signify that not all gene segments are shown with boxes.

Immunoglobulin function

Ig effector functions, are partly determined by the Ig Fc region. The effector function depends on the isotype ability to i) bind specific Fc-receptors (FcR) on cell surfaces, ii) activate the complement system and iii) form oligomers (see Table 2). The organization of the isotype determining constant gene segments of the IgHC locus is shown for mouse and human in Fig.3. IgM is expressed on the surface of almost all mature B cells, with the coexpression of IgD. μ and δ genes are both transcribed from the VH promoter, so RNA splicing will yield either IgM or IgD. The process of isotype switching, which yields other isotypes than IgM/IgD, involves a process of irreversible DNA recombination (1). Isotypes have distinct structures, biological activities and distributions in the body, listed in Figure 4 (structural differences have been simplified). IgM and IgA are usually present as mulitmers in association with the polypeptide J-chain. The different isotypes also vary in positioning and number of linking of disulfide bonds, and degree of amino terminal (N)-linked carbohydrate groups (1).

The Ig Fc/FcR interaction, is an important mechanism mediating Ig effector function. For example, IgG antibodies (Abs) bind the FcRγ on macrophages and neutrophils, mediating phagocytosis of opsonized microorganisms and/or secretion of cytokines involved in inflammation. IgE Abs induce the release of chemical mediators from mast cells and basophils by binding to the FcRε (Fig.4) (1). Though FcRs able to bind IgM have been characterized in humans (100) and mouse (101), and also Fc receptors for IgA in humans (102), these receptors are not as well characterized as FcRs for IgE and IgG. There is also recent report of possible receptors for IgD in humans (103).

IgG is the only Ig capable of crossing the placenta giving immunity to the human fetus, by means of the neonatal FcR (FcRn) (1).

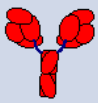
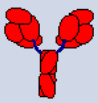
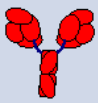
Isotype	IgM	IgD	IgG (G1, G2, G3, G4)	IgA	IgA2	IgE
Structure						
Binding to Fc receptor	+*	-*	+, -, +, +, respectively	+	+	+
Effector functions	Activates complements pathway, opsonization, FcR mediated endocytosis	Activates Basophills and mast cells, triggers cytokine production	Activates complement pathway opsonization, FcR mediated endocytosis ADCC	Activates monocytes and granulocytes opsonization, FcR mediated endocytosis	Activates granulocytes and monocyte opsonization, FcR mediated endocytosis,	Mast cell degranulation, ADCC by Eosinophils
Serum level (mean adult mg/ml)	10	0.03	9, 3,1, 0.5 respectively	3	0.5	$5x10^{-5}$

Figure 4. Structure, characteristics and effector functions of Ig isotypes in human.
Igs are composed of dimerized heterodimers of heavy and light chains.IgM and IgE lack the hinge region that provide flexibility, but they have an additional heavy chain domain. * = less characterized, see text. ADCC = Antibody dependent cell cytotoxicity.

Idiotypes and the idiotypic network

Individual Ig molecules carry unique V region antigenic determinants that are called idiotopes (104-106). In 1971 Sirinsinha and Eisen demonstrated that Ig from the MOPC 315 plasmacytoma was immunogenic: when syngeneic BALB/c were immunized with this Id^+ Ig (in complete Freund's adjuvant (CFA)), they observed an anti-idiotypic Ig response (106). To test if an antigen response in addition to generating antigen specific Abs, also would generate anti-idiotypic Abs to the antigen specific Abs, Rodkey performed following immunizations: first rabbits were immunized with hapten/carrier, then after purifying hapten specific Abs from serum, these were used to immunize the same rabbits again (107). Rodkey found that from the second immunization, anti-anti-hapten Abs resulted. In the wake of such findings, N.K Jerne postulated that the immune system functions as a network based on idiotypic interactions between lymphocytes expressing

complementary BCR (108). Jerne suggested that the network was maintained in a steady state due to a combination of stimulatory and suppressive interactions between clones. In this regard immunizing with Ig as above, would perturb the network.

A corollary of the network hypothesis, is that an Ab1 will through the anti-idiotypic Ab2 generate Ab3, which mimicks Ab1. This was tested and confirmed by Jerne (109). Firm evidence was given for these types of idiotypic cascades in the course of an immune response (110-112). These findings drew scientific attention across various fields, e.g several publications describing the idiotypic network with mathematical models were published in the 1970s and 1980s (113-116).

Adding complexity to the idiotype network of BCR reactive Abs, T cells also express TCR idiotypes and thus they could be regulated by binding to other T cell idiotypes, or B cell idiotypes. Such suggestions were prompted by several experiments in the 1970s and early 1980s (reference only here given for a select few): (117-119). This would imply complex networks where B-B, T-B and T-T interactions are possible (120,121). The significance of such complex connectivity models lost much relevance when the role of the MHC molecule in initiating immune responses became clear.

However the striking and robust phenomena observed in early studies of idiotypic networks have relevance to studies performed decades later. Recent reports show that the idiotypic-connectivity or the disturbed idiotypic-connectivity may play a role in autoimmune diseases (122-125). Further, the concept can be used to modulate the immune system and thereby treat disease, as shown for non small lung cancer (126) and autoimmune disease (127).

The initial event in the pathogenicity of autoimmune disease is still not clear. It is possible that idiotype network may play a role, especially when considering Th cell-B cell interactions, see the discussion.

T lymphocytes, development and function

T cell development

The crucial events in T cell development occur in the thymus. Important subset-defining T cell surface receptors are CD4, CD8 and T cell receptor complex molecules (CD3, and T cell receptor αβ chains). Immature thymocytes (T cells precursors) do not express these markers. Thymocytes may give rise to a minor $\gamma{:}\delta^+$ T cell subpopulation or $\alpha{:}\beta^+$ T cells. Thymocytes go through a step in which they express a preTCR, and after division become small resting $CD4^+/CD8^+$ double positive (DP) (1). 95% of these cells undergo apoptosis in the thymus (128).

The DP cells that express TCR that bind MHC class II/I, receive survival signals, migrate into the thymic cortex and differentiate into mature T cells (1,129). Strongly self-reactive clones undergo apoptosis in a process termed negative selection (128). However, not all T cells with high self affinity are removed: it has been suggested that natural regulatory T cells require high affinity TCR binding (130). Negative selection is controlled by many molecules acting in concert (131,132).

Weakly self reactive T cells are positively selected. Positive selection is aided by the CD8/CD4 molecules. According to the instructive model, DP cells recognize MHC class I-peptide, engage CD8 and downregulate CD4. Reciprocally, DP cells that recognize MHC class II-peptide, engage CD4 and downregulate CD8. The surface molecules TCRα-CPM, CD3γ-ITAM, CD3δ are essential in initial positive signaling (131).

Elimination of T cells in the thymus is termed central tolerance. T cells are also subject to peripheral tolerance. Peripheral T cells that are chronically exposed to MHC/peptide, will become anergic and non-responsive if costimulatory signals or inflammatory cytokines are lacking (1). Regulatory T cells also play a very important part in peripheral tolerance (133-135).

$CD8^+$ cytotoxic T cells

Naïve $CD8^+$ T cells that undergo priming in peripheral lymphoid organs through the MHC class I/peptide complex on APC, will differentiate into cytotoxic effector T cells. The priming of *Naïve* $CD8^+$ T cells may require assistance by T helper cells (136). Cytotoxic T cells serve to monitor cells and tissue for viral antigen, and induce apoptosis of cells that present MHC class I/peptide (1).

CD4+ T helper cells

Naïve $CD4^+$ T cells that that are primed with peptide/MHC class II on APC, will differentiate into one of the T helper subsets.

Th1 and Th2

The classical T helper subsets are Th1 and Th2 (1). The manner that the innate immune system responds to an invading pathogen defines the cytokines that may impact T cell priming and the outcome of the T cell polarization. It is commonly accepted that immunological responses to intracellular pathogens (virus and some bacteria) favors Th1 polarization, while responses to extracellular pathogens (e.g worms and allergens) favors Th2 polarization (1). It has also been suggested that the strength of the interaction of TCR with antigen/MHC may play a role (137).

Th1 cells promote inflammatory innate immune responses by supporting macrophages and the production of opsonizing Ig subclasses in B cells. Th1 are charcterized by production of IFNγ. IFN-γ activates macrophages, inhibits Th2 development, supports antigen processing and increases MHC expression on APCs (1).Th1 cells also express Fas ligand (The Fas ligand-Fas interaction induces apoptosis of Fas expressing cells) (1). For Th1 effector functions, transcription factors (TFs) and cytokines, see Figure 5.

Th2 cells express the GATA3 transcription factor and are important in the adaptive immune response. Th2 are specialized for B cell activation: they produce IL-4, IL-5, IL-13and IL-15 that are potent B cell stimulatory factors. The Th2 cytokines IL-10 and TGFβ inhibit Th1 polarization (1). For Th2 effector functions, transcription factors and cytokines, see Figure 5.

Th17

Th17 play an important role in clearance of extracellular pathogens, especially at mucosal surfaces (138). It is believed that they play a key role in localized focal autoimmune diseases (139,140). Several factors have been identified as being involved in Th17 development; TGFβ, IL-6, IL-21, IL-23 and transcription factors STAT3, RORγT and RORα, as reviewed in (138). See Figure 5 for Th17 effector functions, cytokines and transcription factors.

T follicular helper (Tfh)

Tfh are a T cell subset involved in the activation of B cells in the germinal center (GC) reaction (see "Th cell - B cell collaboration"). Tfh cells express high levels of the chemokine receptor CXCR5 which contributes to the co-localization of Tfh and B-cells in follicles (141,142). Tfhs require IL-21 for generation (143). The transcriptional repressor Bcl-6 has been used to distinguish the Tfh subset from other Th cell subsets. Moreover, Tfhs are PD-1^{high} and $ICOS^{+}$ (144). See Figure 5 for further details.

Regulatory T cells, T_{regs}

T_{reg} cells are important in maintaining immune homeostasis, and preventing detrimental T cell responses (133).Their dysfunction may be involved in autoimmune disease, immune pathology and allergy (145). The T_{reg} subset is commonly distinguished by the forkhead transcription factor (FOXP3) expression (146). The two major classes of FOXP3 T_{regs} are classified as $CD4^{+}/CD25^{+}$ naturally occurring T_{regs} (nT_{regs}), originating directly from thymus, and T_{regs} derived from primed naïve Th cells under the influence of TGFβ. This latter T_{reg} type is called induced T_{regs} (iT_{regs}) (147). See Figure 5 for further details.

T helper subset plasticity

There is some plasticity to the profile of T helper subsets. Th17 cells may convert to Th1 cells (148). Th2 can also become IL-9 secreting cells (adapting a Th9 profile, not addressed herein) (149). Also Th2 cells have been shown to transform into $CXCR5^{+}$ Tfh cells (150). $FOXP3^{+}CD4^{+}$ T cells have also been shown to differentiate into Tfh in peyer's patches (151), thus becoming an effector cell no longer suppressing but promoting immune responses.

The classical view has been that T helper lineage commitment is unidirectional and fixed for Th1- and Th2 cells, and that these express a unique cytokine and TF profile. With the discovery and characterization of new subsets such as T_{reg} and Th17, the classical view is being reshaped. An overview of Th lineage development and characteristic factors and functions is given in Figure 5.

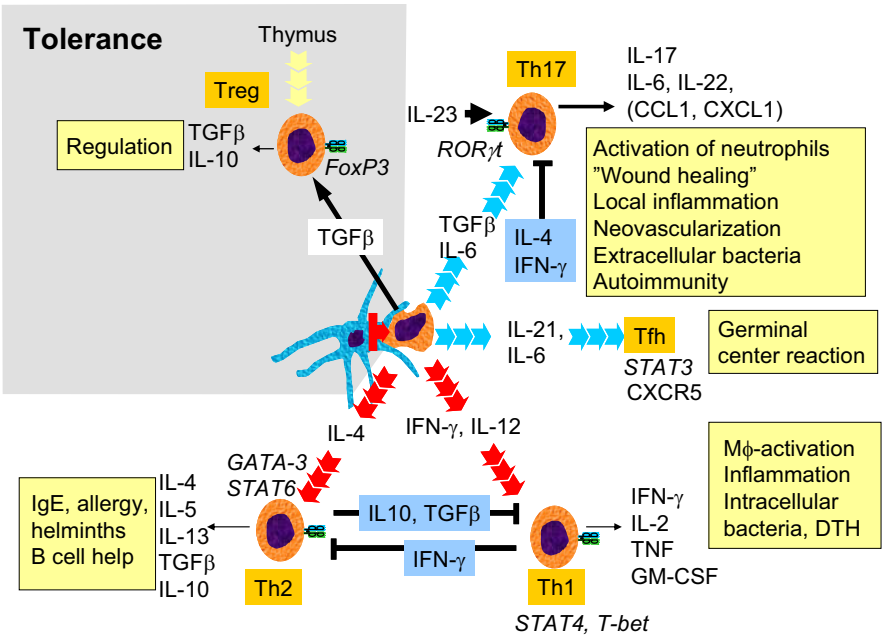


Figure 5: Development and characteristics of the $CD4^+$ T cell lineage. When primed by APC in the periphery, naïve $CD4^+$ T cells, depending on help from the cytokine environment, differentiate into one of five subclasses: either the classical Th1, Th2 or the more recently discovered Th17, Tfh and induced T_{reg} (iT_{reg}). Transcription factors are given in italics

Cytokines

The cytokine environment is of crucial importance for adaptive and innate immune functions. Cytokines effect lymphoid proliferation and survival, antigen presentation and trafficking in cells, and organogenisis of lymphoid organs. Moreover, the cytokines interface with peptide hormones of the central nervous system (1). An updated list of cytokines, with their source and function, is given in Table 2.

Cytokine	Source	Function
IL1-α and -β	macrophages and other antigen presenting cells (APCs)	co-stimulation of APCs and T cells, inflammation and fever, acute phase response, hematopoiesis
IL-2	activated Th1 cells, NK cells	proliferation of B cells and activated T cells, NK functions
IL-3	activated T cells	growth of hematopoietic progenitor cells
IL-4	Th2 and mast cells	B cell proliferation, eosinophil and mast cell growth and function, IgE and class II MHC expression on B cells, inhibition of monokine production
IL-5	Th2 and mast cells	eosinophil growth and function
IL-6	activated Th2 cells, APCs, other somatic cells such as hepatocytes and adipocytes	acute phase response, B cell proliferation, thrombopoiesis, synergistic with IL-1β and TNF on T cells
IL-7	thymic and marrow stromal cells	T and B lymphopoiesis
IL-8	macrophages, other somatic cells	chemoattractant for neutrophils and T cells
IL-9	T cells	hematopoietic and thymopoietic effects
IL-10	activated Th2 cells, $CD8^+$ T and B cells, macrophages	inhibits cytokine production, promotes B cell proliferation and antibody production, suppresses cellular immunity, mast cell growth
IL-11	bone marrow stromal cells	synergisitc hematopoietic and thrombopoietic effects
IL-12	B cells, T cells, macrophages, dendritic cells	proliferation of NK cells, INF-γ production, promotes cell-mediated immune functions
IL-13	Th2 cells, B cells, macrophages	stimulates growth and proliferation of B cells, inhibits production of macrophage inflammatory cytokines
IL-14	T cells and malignant B cells	regulates the growth and proliferation of B cells
IL-15	virus infected macrophages, mononuclear phagocytes	induces production of NK cells
IL-16	eosinophils, $CD8^+$ T cells, lymphocytes, epithelial cells	chemoattractant for $CD4^+$ cells
IL-17: six isoforms all from different genes; IL-17A, B, C, D, E, and F (IL-17E also called IL-25)	A and F forms only expressed in a subset of T cells; B expressed in leukocytes and peripheral tissues; C up-regulated during inflammation; D expressed in nervous system and skeletal muscle; E expressed in peripheral tissues	increases production of inflammatory cytokines, angiogenesis, affects endothelial and epithelial cells
IL-18	macrophages	increases NK cell activity, induces production of INF-γ
INF-α and -β	macrophages, neutrophils and some somatic cells	antiviral effects, induction of class I MHC on all somatic cells, activation of NK cells and macrophages
INF-γ	activated Th1 and NK cells	induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells, activates macrophages, neutrophils, NK cells, promotes cell-mediated immunity, antiviral effects

Table 2. Cytokines; source and effect in the immune system. Adapted from http://themedicalbiochemistrypage.org/, Michael W. King, PhD/IU School of Medicine.

Th cell - B cell collaboration

Th cell - B cell collaboration is a central part of the adaptive immune response (humoral immunity). In a typical immune response, B cells and $CD4^+$ T cells (T follicular helper cells, Tfh) interact in the germinal center (GC) of peripheral lymphoid organs to produce somatically mutated, high affinity, predominantly isotype switched Ab (152) that aid in the clearance of pathogens. This response persists partly through secretion from long lived plasma cells (153). Another outcome of this Th cell - B cell collaboration is the formation of long lived memory B cells (154), enabling the rapid response upon re-exposure to antigen by differentiating into plasma cells (155).

Historical milestones towards our understanding of T cell -B cell collaboration

Key observations on Th cell - B cell collaboration, were done in the 1960s, where thymectomy inhibited Ab production, thereby demonstrating the importance of T cells (156). Transfer of B cells (bone marrow cells) and T cells (thymus cells) into irradiated hosts showed that both were necessary for Ab formation: single transfers abrogated Ab production (157). Simultaneously the role of "carrier proteins" in Ab production was being elucidated. Haptens are unable alone to mount Ab responses, but can do so when coupled to so-called carrier proteins. Transfer experiments demonstrated that spleen cells from donors immunized with the carrier protein bovine serum albumin (BSA), when transferred into another mouse of the same strain, help co-transferred lymphocytes, identified as thymus derived, from an animal immunized with 4-hydroxy-3-iodo-5-nitrophenylacetitc acid (NIP)-conjugated to ovalbumin, to produce NIP specific antibodies when immunized with NIP-BSA (158,159). Earlier experiments also demonstrated that in the immunization with hapten conjugates, it was a requirement for Ab responses that the antigen had more than one antigenic determinant (160). In summary, antigenic determinants (epitopes) recognized by T and B cells must form part of the same structure. This led to the concept of an "antigen bridge" that was necessary for antibody production. When the T cell marker Thy1 was discovered, the marker was used to definitely show that the carrier specific population were T cells (161,162). These studies were followed by the discovery of the role of MHC class II molecules (163), the cloning of the TCR and the demonstration of peptide loading onto MHC molecules of B cells (164), largely progressing our understanding of the mechanisms of Th cell – B cell collaboration.

Events leading to the GC formation

T dependent B cell memory requires $CD4^+$ Th cell and B cell contact in GCs within the follicles of lymphoid organs (165). Initial events leading to the GC formation are summarized in Figure 6. T-dependent Ab responses are initiated when rare B and Th cells specific for an antigen interact in the T cell - B cell boundary (166). Thereafter, the B cells either move into the extra follicular areas as short lived plasma cells or migrate into the GC (167).

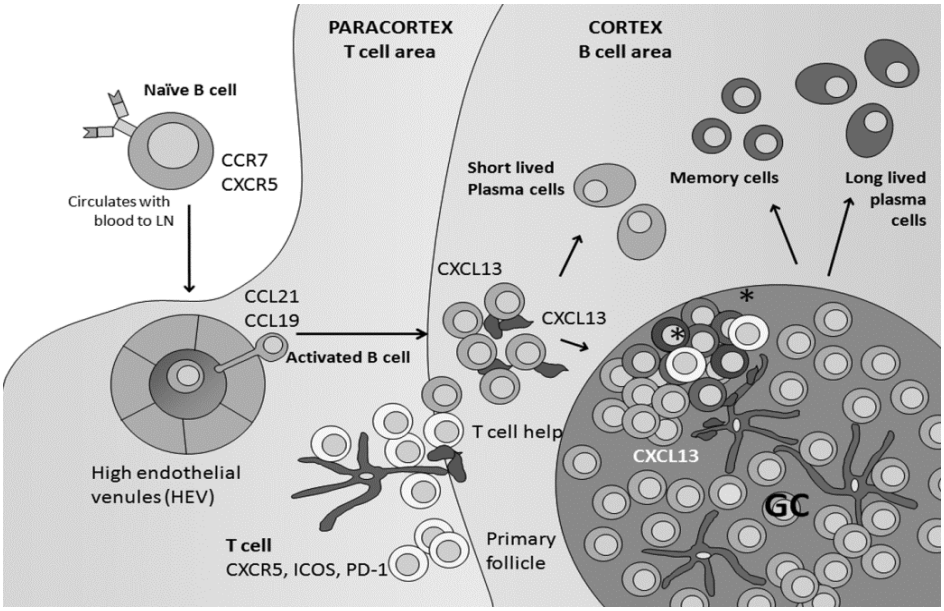


Figure 6. Events leading to the formation of germinal centers. Naïve B cells enter the lymphoid organ through High endothelial venules (HEV). The entering B cells will migrate in the follicle dependent on expression of chemokine receptor CXCR5. CXCR5 is expressed on all mature B cells (168) and its ligand CXCL13 is made by follicular stromal cells in the subcapsular region of the follicle and follicular dendritic cells (FDCs) in the center of the follicle (169,170). Naïve B cells also express CCR7, the receptor for CCL21 and CCL19. CCL21 and CCL19 are expressed in T cell zones (171) and CCL21 extends into lymphoid follicles in a decreasing gradient (172). Antigen specific T cells primed on dendritic cells in the T cell zone upregulate ICOS, PD-1 and CXCR5 and migrate towards the T-cell/B-cell interface zones. In areas forming primary foci, T cells can interact with B cells. If a B cell receives cognate T cell help, there will be a proliferative burst. At his point some B cells are fully activated and secrete mainly IgM. Other B cells will upregulate CXCR5 and migrate according to the increasing gradient of CXCL13 and form GC B cells. Antigen specific T cells primed on DC in T cell zone, upon interaction with a cognate B cells becomes Thfs. T cells upregulate CXCR5 to enable follicular localization.* = this interaction will be elucidated. GC B cells emigrate from the follicle and differentiate into long-lived plasma cells and memory B cells.

The germinal center

Contrary to previous dogma, it has been revealed that the germinal center is an open and dynamic organ, where both antigen-specific and non-specific B cells may enter (173).

The nature of this process has not been completely understood, however it is believed that high affinity BCR cross-linking favors plasmablast formation (174). Also the long-lived Th cell - B cell cognate contacts may be fate determining (175). As the GC matures two compartments become distinct. These compartments were first described based on their histological appearance (176); the light and the dark zone. GC B cells in the dark and light zone were classified as centroblasts and centrocytes respectively, each with specific characteristics (177). Real-time imaging has lately given a lot of new data, and challenged many of the previous ideas (173). The GC reaction as it takes place in the light zone and the dark zone has been summarized in Figure 7. In the light zone Follicular Dendritic Cells (FDCs) accumulate, with distinct features from FDC in primary follicles; upregulated VCAM-1 and FcγRIIB (178,179). The light zone seems to be positioned towards the source of foreign antigen (180) and antigen is transported rapidly to the light zone (181). CXCL13 is more abundant in the light zone, and CXCL/CXCR5 is necessary for accumulation of GC B cells to accumulate in the light zone (182). The chemokine receptor CXCR4 is needed for GC B cells to locate to the dark zone and its ligand SDF-1 is more abundant in the dark than light zone (182). As for Tfh cells, they are much more abundant in the light zone than the dark zone (183) and are characterized by expression of CXCR5 and ICOS (183). Tfh cells interact preferentially with B cells displaying the highest levels of MHC class II and induce them to move to the dark zone for proliferative expansion (184). GCs can form independently of cognate interactions with Th cells, but these GCs collapse shortly after compartmentalization into dark and light zones (185). GC B cells have been estimated to be 5-20 times more abundant in GCs than T cells, but less than one third of T cells were moving at a speed permissive of T cell - B cell conjugates (181). This suggests that there would be high competition of GC B cells for cognate Th cell help. This competition for Th cell help has been suggested as an effective mechanism of achieving high affinity clones (186).

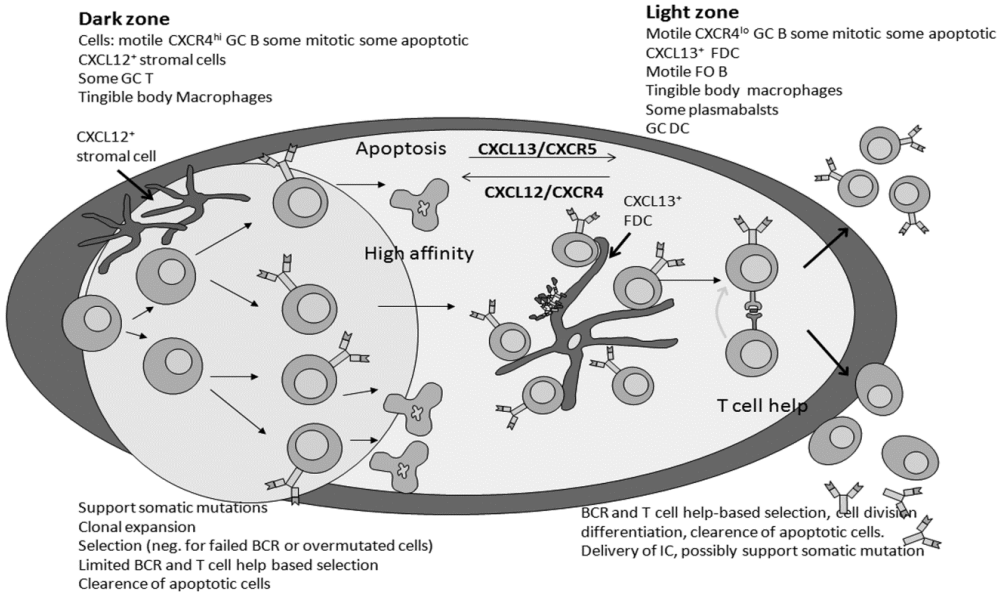


Figure 7. Cellular composition and function of dark and light zones in the germinal center (see text for references). GC B cells accumulate in the dark zone after mitosis. Somatic mutation may also occur here. After mitosis, B cells down-regulate CXCR4, cells can leave the dark zone and enter the light zone by a CXCL13 gradient. Here, the B cells move around the FDC network, where the B cells could receive survival signals and also endocytose, process and present Ag removed off the surface of FDCs. Subsequently B cells would compete for Th cell help. Cells entering the light zone undergo apoptosis, exit the GC or return to the dark zone. Cells exiting the GC differentiate into long-lived plasma cells or memory B cells. Tingible body macrophages are macrophages that have engulfed the nuclei of dead B cells (187).

Molecular interactions between Tfh and GC B cells and downstream events

Antigen specific Th cells primed on DCs in the T cell zone, may up-regulate ICOS, PD-1 and CXCR5 and migrate towards B cell follicles, where an interaction with B cells (in a IL-21 associated process) may allow cells to express Bcl-6 and become Tfh cells. Certain molecules have been shown to be a requirement for the Tfh - B cell interaction in the GC. ICOS and CD40L are absolutely required for Tfh cell differentiation and thus GC development (188). CXCR5, the adaptor SAP and cytokines such as IL-21 and IL-4 are partially required for Tfhs. GCs that arise in their absence are in varying ways defective (142,154,189). The absence of IL-27 receptor has a detrimental effect on Tfhs as they do not expand normally and produce less IL-21 (190). Some of the important molecules in the Tfh - GC B cell interaction are given in Figure 8.

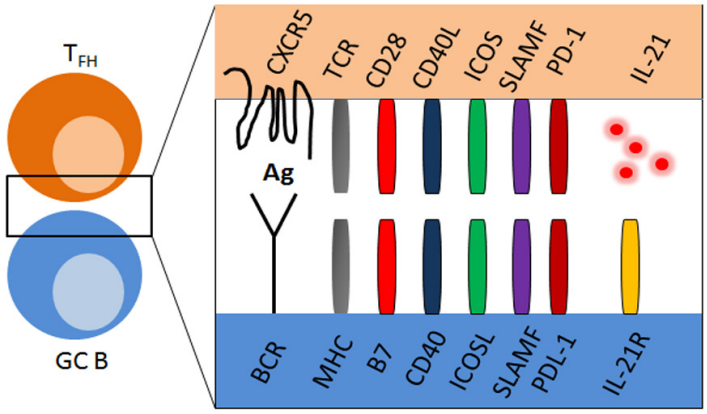


Figure 8. TheTfh - B cell molecular interaction in the GC. Molecular pairings culminate in the T cell secreting cytokines, particularly IL-4 and IL-21.

Conventional ("linked") vs non-conventional ("non-linked") Id-driven Th cell - B cell Collaboration

The previously introduced concept of the "antigen bridge" in T cell - B cell collaboration was later termed "linked" T cell – B cell collaboration (191). However interactions are possible that do not conform to this norm. For example, in mixed lymphocyte reactions, alloreactive Th cells may be activated by allo-MHC, while B cells may be specific for unrelated antigen. In such Th cell- B cell collaboration, the interaction is not linked to a common antigen. Another example of non-linked Th cell – B cell collaboration was named "non-linked" Id-driven Th cell – B cell collaboration. For simplicity Th cell – B cell collaboration will be termed T cell – B cell collaboration.

To explain this interaction, some premises must be presented: Our group (78,79,192) and others (193) have shown that B lymphoma cells (78,79), B cells from transgenic mice (192) and normal B cells (193) present BCR V region Id in a MHC class II dependent fashion to Id-specific Th cells, resulting in T cell proliferation and in turn B cell differentiation and Ab secretion (193,194). This Id-driven T cell - B cell collaboration is non-linked in the sense that the B cells and T cells do not recognize epitopes on the same antigenic entity. This chronic Id-driven T cell - B cell collaboration can lead to B cell lymphoma development (195) and autoimmune disease manifestations (196).

The “non-linked Id-driven” T cell - B cell collaboration described above, has been investigated in our group using the The λ^{2315} model. This model has also been the basis for experiments reported in this thesis.

The λ^{2315} model

The Id-specific Th cells in our experiments, are Th cells specific for the λ^{2315} T cell epitope of the myeolma protein M315. The λ^{2315} model has been employed extensively in our group to study the above mentioned Id driven “non-linked” Id driven T cell –B cell collaboration. The model system and all its components are given in Figure 9.

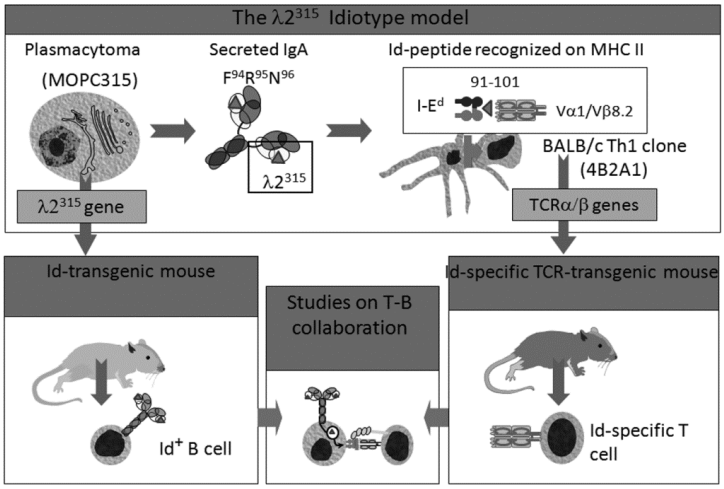


Figure 9. The secreted M315 IgA mAb (referred to as Id^+ in our studies) of the MOPC315 plasmacytoma cell that arose in BALB/c after injection of mineral oil i.p (197), has three mutated aa in positions 94,95 and 96 of the V gene region of λ LC (198). Id-specific $CD4^+$ T cells from BALB/c mice immunized with λ^{2315}, that recognized a CD3 peptide spanning aa 91-101 in a MHC class II ($I\text{-}E^d$) restricted fashion were cloned (199-201). TCR α-and β genes of the T cell clone 4B2A1 were used to generate TCR transgenic mice (202).

Apoptosis in a B cell tolerogenic perspective

Autoreactive B cells can be deleted as one of several tolerogenic mechanisms (see general discussion). In the periphery, self reactive B cell clones can arise from Th cell dependent somatic hypermutation (203). Such clones would require elimination to avoid autoimmunity (204). Deletion of peripheral B cells with BCR specific for a membrane bound self-antigen has been demonstrated with transgenic mice (205). It has been suggested that for immature B cells in the bone marrow, the microenvironment supplies signals that favor receptor editing for autoreactive B cells, where the lack of these signals in the periphery would direct towards apoptosis (206). However also immature autoreactive B cells can undergo activation induced cell death (AICD) (207,208).

The role of caspases in AICD

BCR mediated apoptosis involves the activation of caspases (CysteineAsparthyl Specific Proteases), a family of cysteine proteases that cleave their substrates at aspartic residues (209). Caspases are classically grouped into the initiator caspases (caspase 2, 8, 9, 10) and the effector caspases (caspase 3, 6, 7). Caspases are regulated at a post-translational level, ensuring that they can be rapidly activated.

Apoptosis has classically been separated into two pathways, one requiring activation of death ligands and receptors (210) and the other involving mitochondria (211). However later evidence shows that they are linked and that molecules can influence each other. A highly simplistic illustration of the two classical caspase activation pathways is given in Figure 10.

BCR induced caspase activation is triggered independently of death receptors and caspase 8 (212). In primary B cells (209) and in mature (213) as well as immature (214) B cell lines, BCR mediated apoptosis has been suggested to be a result of the intrinsic apoptosis pathway of cytochrome c/Apaf-1/caspase-9, connecting the mitochondria to executioner caspases (215).

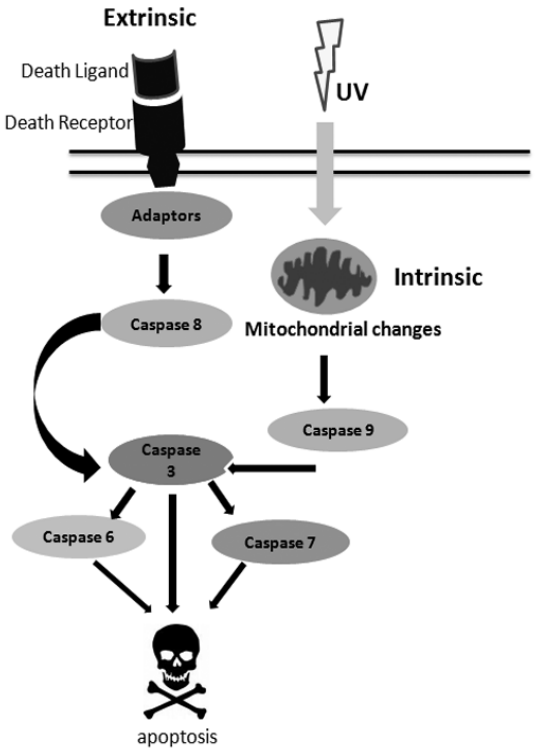


Figure 10. Pathways for caspase activation: The extrinsic pathway, connects the cell- surface binding ligand to apoptosis induction by the Tumor Necrosis Factor (TNF) family of cytokine receptors such as TNFR and Fas (216), depicted as death ligand/receptor. The intrinsic pathway involves the participation of mitochondria (e.g induced by UV radiation), releasing caspase activating proteins into the cytosol and finally triggering apoptosis (217). Active caspase 8 and 9 have been shown to directly cleave and activate the effector protease caspase 3. Effector caspases 6 and 7 are only two of several effector caspases. The figure shows only a very simplified overview of the two main pathways for caspase activation.

AIMS OF THE STUDY

To this day, the impact of the idiotype networks on the immune system is unclear. As advances in molecular biology have provided new tools, we can now use mouse models to study the basic mechanisms of idiotypic regulation under physiological conditions.

Firstly we wanted to investigate how idiotype$^+$ antibodies (Id$^+$ Ab) and anti-idiotypic antibodies (anti-Id Ab) could communicate in the context of interactions that also involved idiotype-specific Th cells. Could a B cell with anti-idiotypic BCR provide an efficient APC for idiotype-specific T cells when presented with idiotype$^+$ Ig ?(paper1). How efficient would this mechanism be under physiological conditions? (paper3)

It is unclear to what extent idiotype network interactions can occur in the absence of T cells. What would happen when B cells with complementary BCR idiotypes interacted? (paper2). Such interactions could potentially shape the early and late B cell compartments.

SUMMARY OF INDIVIDUAL PAPERS

Paper 1: The cellular mechanism by which complementary Id^+ and anti-Id antibodies communicate: T cells integrated into idiotypic regulation

Johanne T. Jacobsen, Elin Lunde, Vibeke Sundvold-Gjerstad, Ludvig A. Munthe and Bjarne Bogen.

In this paper we wanted to elucidate how Id-specific Th cells and anti-Id B cells presenting Id^+ Ig, communicate. The issue is pertinent as the idiotypic connectivity in an individual to this day lacks a mechanistic explanation. Both in *vitro* and *in vivo* models were used: The *in vitro* model was B lymphoma cells expressing an anti-Id BCR (IgD, only membrane bound form), in context of Id-specific Th cells and Id^+ Ig. The *in vivo* models were i) a surrogate model using Id-specific T cells, a recombinant Ig, harboring the Id^+ peptide, directed to IgD^a (anti-IgD^a rIg) and BALB/c (IgH^a) B cells, all transferred to C.B-17 (IgH^b) recipient mice and ii) anti-IgD^a recombinant Ig delivered to TCR transgenic mice (harboring Id-specific T cells).

The *in vitro* model described above showed that the anti-Id B cells were $x10^4$ more efficient at presenting Id^+ Ig to $CD4^+$ Th cells. The anti-Id BCR was required for proliferation of Id-specific Th1/Th2 and effector functions of Id-specific Th1 cells in the presence of Id^+ Ig. The surrogate *in vivo* models i) and ii) demonstrated that B cell proliferation and generation of anti-Id Abs was highly dependent on Id^+ being targeted to the BCR (IgD).

Conclusions: Our results demonstrated how Id^+ Ig was efficiently presented to Id-specific T cells by anti-Id expressing B lymphoma cells, thus activating the Th cells. The results further demonstrated that this idiotypic Th cell - B cell collaboration also applied to normal B cells *in vivo,* with Id^+ being delivered to the BCR. The results of this paper were unphysiological. Hence, further investigations were merited in more physiological settings where Id^+ Ig could be presented to Id-specific Th cells by normal B cells through bona fide Id^+/anti-Id interactions.

Paper2: B lymphoma cells with mutually binding B cell receptors kill each other: a mechanism for reduced idiotypic connectivity?

Johanne T. Jacobsen, Vibeke Sundvold-Gjerstad, Frode M. Skjeldal, Oddmund Bakke, Anne Spurkland and Bjarne Bogen.

As an inevitable consequence of antibody diversity, B cells with complementary BCRs (defined by their respective idiotypes) will at some time interact. This could happen either in the bone marrow or in the peripheral lymphoid sites. To our knowledge, this issue had not been studied. In order to investigate the issue, we resorted to using B lymphoma cells in a model system. We generated A20 B lymphoma cells expressing complementary sets of BCR, enabling Id^+/anti-Id interactions ("anti-Id" A20 was generated in paper 1). To eliminate the confounding effect of secreted Ig, we restricted the idiotypes to membrane expression by excising the secretory exons. In a range of different assays we demonstrated that Id^+/anti-Id (BCR/BCR) interaction of these cells resulted in apoptosis. Both cell types were prone to apoptosis, but overall the Id^+A20, with a lower BCR density than anti-Id A20 was more susceptible. Also, in conjugates of Id^+/anti-Id B cells, it appeared that only one cell would receive an apoptosis signal (caspase activation). The Id^+/anti-Id BCR/BCR interaction was also compared to Id^+/anti-Id BCR/Ig interactions (for both Id^+A20 and anti-Id A20). We observed that the BCR/BCR interaction was more efficient at inducing apoptosis. We also observed that apoptosis induction with Ig/BCR adhered to conventional activation induced apoptosis: a higher BCR density conferred increased apoptosis.

Conclusion: Our results suggested that the Id^+/anti-Id BCR/BCR induced apoptosis could be an efficient tolerance mechanism restricting the B cell repertoire. The outcome could depend on many factors such as: BCR density, Id^+/anti-Id affinity, activation state and subset of the B cell and amount of secreted Ig. A physiological model to further investigate the mechanism is not currently available.

Paper 3: Anti-Id B cells and Id-specific $CD4^+$ T cells collaborate efficiently under physiological conditions.

Johanne.T Jacobsen, Ludvig. A Munthe, Karoline W. Schjetne and Bjarne Bogen.

Moving towards a more physiological situation and answering some of the issues described above, we here used a novel Ig receptor knock-in mouse. This mouse was generated in the current work. The anti-Id Ig knock-in mouse on a BALB/c background (resulting from a cross of anti-Id IgH knock-in and anti-Id IgL knock-in) expressed the anti-Id BCR on almost all B cells. The B cells demonstrated enhanced development in bone marrow, most likely due to the pre-rearranged BCR. Anti-Id mice demonstrated a normal B cell subset distribution in the periphery. There were no signs of an abnormal phenotype in this mouse. Using B cells from this knock-in mouse with Id-specific $CD4^+$ T cells and Id^+ Ig, we demonstrated (*in vitro* and *in vivo*), that the "linked" idiotype-specific T cell - B cell collaboration was highly efficient. We demonstrated that this specific interaction was initiated even with naïve T cells, and that low numbers of cells and small amounts of Ig were sufficient to elicit responses. *In vivo,* anti-Id isotype switching and GC formation was observed.

Conclusion: Our findings in this paper offer a physiological model explaining the basic mechanism of idiotypic connectivity. Our results suggest that small numbers of anti-Id B cells and Id-specific Th cells can interact in presence of low concentrations of Id^+ Ig, in an unprimed physiological setting.

METHODLOGICAL CONSIDERATIONS

Transgenic mice (paper 1 and 2)

Id-specific TCR transgenic BALB/c and TCR transgenic SCID (CB.17 scid/scid) (paper 1 and 3)

A TCR transgenic (TCR TG) mouse was generated from the TCR α and β genes (Vα1, Jα19; Vβ8.2, Jβ1.2) of the T cell clone 4B2A1 (202). This TCR TG mouse has $CD4^+$ T cells responding to the $\lambda 2^{315}$ light chain of the MOPC 315 protein M315 in a MHC class II restricted manner. Both TCR TG BALB/c (202) and TCR TG SCID (*CB.17 scid/scid*) (218) were used in our experiments.

The TCR TG BALB/c holds totally lower numbers of cells in spleen and lymph nodes compared the non TG BALB/c. However, TCR TG BALB/c Id-specific T cells seem to have a normal phenotype. TCR, CD4 and CD8 density on peripheral T cells of TCR TG and normal BALB/c do not differ (202). TCR TG SCID are unable recombine endogenous TCR or BCR genes (due to a defect in the DNA-dependent protein kinase catalytic subunit (219)), thus the number of cells in lymphoid organs is severely reduced. However, also TCR TG SCID show a normal expression of TCR, CD4 and CD8 on peripheral T cells (218).

In TCR TG SCID the allelic exclusion of endogenous α and β chains is complete (218). However for T cells in TCR TG BALB/c, only a fraction express the transgenic α, whilst almost all express the transgenic β (202). This is probably due to preferential αβ pairing (220). The Id-specific T cells in TCR TG BALB/C that do express the transgenic α, can also co-express endogenous α, due to functional rearrangements. These cells make up the main fraction of Id-specific T cells in the TCR TG BALB/c and stain dimly with the clonotype specific mAb GB113 compared to Id-specific T cells from TCR TG SCID, which express only transgenic αβ (218). The double specificity Id-specific T cells (expressing endogenous α and transgenic α) in TCR TG BALB/c, have a reduced signaling capacity and response to $\lambda 2^{315}$/I-E^d compared to Id-specific T cells expressing only transgenic α (221). The signaling capacity of the Id-specific T cells is a concern, especially in paper 3, where we tried to determine the lower threshold of cells and Id^+ Ig needed to elicit Id^-specific B cell and T cell responses. The anti-Id B cell/Id-specific T cell responses in the presence of Id^+ Ig, could in fact be more sensitive with Id-specific T cells from TCR TG SCID. TCR TG SCID were used in the surrogate model, paper 1.

Id-specific T cell - B cell interactions if naturally occurring, could include either a mono- or bispecific T cell as 15-30% of peripheral T cells normally have dual α expression (222,223).

To exclude the possibility of Id-specific $CD8^+$ T cells confounding our results, these were always removed when lymph node (LN) cultures from TCR TG BALB/c were used *ex vivo*. In paper 1, both TCR TG BALB/c and TCR TG SCID were used as recipients. For TCR TG BALB/c, we cannot exclude that Id-specific $CD8^+$ T cells had a minor dampening effect on the recorded responses, due to cross presentation. However for TCR TG SCID the number of Id-specific $CD8^+$ T cells are severely reduced compared to TCR TG BALB/c (218), so the effect would be negligible.

Anti-Id ^{DKI} (paper 3)

Unlike the TCR TG mice described above, the anti-Id BCR mouse generated in paper 3, is a targeted transgenic: rearranged Ig genes are targeted to positions in their native Ig loci. With the insertion of a rearranged VDJ or VJ gene sequence into its physiological position in the Ig locus, we obtained a single gene copy under the control of endogenous cis-acting DNA elements that naturally control expression.

The HC VDJ anti-Id gene sequence was inserted into the Ig locus, by exchanging it with the unrearranged DQ52J region (essentially as previously performed (224)). In doing so, all endogenous J genes were removed. In theory this exchange of gene sequences should exclude endogenous rearrangements, as recombination signal sequences (RSS) were removed (although a rare endogenous rearrangement using cryptic RSS in the inserted V gene could occur (225)).

For LC VJ anti-Id, our strategy was targeting the VJ of anti-Id into the κ locus, but leaving the endogenous Jκ 3-5 intact (essentially as previously performed (226)). This would in theory not exclude endogenous rearrangement from upstream Vκ genes. However, this was not a major event, since in our anti-Id double knock-in (anti-Id ^{DKI}) almost all peripheral B cells expressed the anti-Id specificity. Even in old mice, almost all B cells expressed the anti-Id BCR. Thus allelic exclusion of endogenous HC and LC Ig genes was efficient and endogenous gene rearrangement probably very low frequent.

The peripheral anti-Id B cells exhibited a normal surface density of BCR, IgM and IgD. The peripheral B cell pool in anti-Id ^{DKI} had a normal B cell subset distribution. The

only abnormal feature observed in the anti-Id ^{DKI}, was an enhanced developmental state of bone marrow B cells, as previously reported (227).

Targeting Id$^+$ to IgD, a surrogate model for T cell - B cell collaboration (paper 1)

In paper 1 we used a surrogate model for Id-specific T cell - B cell collaboration. In this model, we used IgD^a-specific recombinant Ig (rIg) with Id$^+$ peptide inserted into the C_H1 domain of human γ3 HC (228). Together with Id-specific T cells and HC allotype a B cells, Id-specific T cell - B cell collaboration could be studied. However this T cell - B cell collaboration is not "linked", as there are no *bona fide* Id$^+$/anti-Id interactions involved.

In vitro, anti-Id A20 with Id$^+$ Ig was x10^4 more efficient at inducing Id-specific Th2 proliferation compared to the non - transfected A20 with Id$^+$Ig (paper 1). It was previously demonstrated that A20, expressing hapten 2,4,6-trinitrophenyl (TNP) specific membrane IgD^a, with Id$^+$ rIg was x10^3 more efficient at inducing Id-specific Th2 proliferation compared to the control A20, expressing TNP specific membrane IgM^a, with Id$^+$ rIg (228). The IgD levels on anti-Id A20 and anti-TNP A20 were only investigated using anti-IgD Abs in flow cytometry (paper 2 and (229)) and not exactly quantified. Thus it is possible that anti-Id A20 has a slightly higher membrane IgD expression than anti-TNP A20, and thus an increased ability to stimulate Id-specific T cells.

In vitro, for normal B cells, anti-Id B cells with Id$^+$ Ig were x10^3 more efficient at inducing Id-specific Th2 proliferation than control BALB/c B cells with Id$^+$ Ig (paper 3). IgD ^{a} B cells from BALB/c, with Id$^+$ rIg revealed a 100-1000 fold increased efficiency at inducing Id-specific Th2 proliferation compared to the control B cell, IgD^b from C.B-17, with Id$^+$ rIg (228).

These results show that both the surrogate model using Id$^+$ rIg targeted to IgD (paper 1) and the bona fide "linked" Id-driven T cell – B cell model (paper 1 and 3) are both efficient at inducing Id-specific T cell proliferation. The M315 mAb (Id$^+$ Ig), is an IgA (197). If Id$^+$ Ig was of another isotype, the anti-Id B cell activation and subsequent Id-specific T cell activation could be even more pronounced: IgM would have high avidity and thus increased ability to crosslink BCR and IgG isotypes could cross-link FcγRs. Or, if the inhibitory FcγRIIb was engaged, B cell activation could be reduced (230).

A20 B lymphoma cells (paper 1 and 2)

A20 cells are easy to transfect and culture. The A20 cell line, as other B lymphoma cell lines has different growth capacities than normal B cells. Thus it could be a concern that the A20 signaling apparatus differs from normal B cells. However, A20 cells have been used as normal B cells in numerous BCR signaling experiments (231-233). A20 B lymphoma cells have also been used to study B cell antigen processing and presentation (228,234). Other murine B lymphoma cell lines have frequently been used to study BCR mediated apoptosis (235,236). So although it would have been preferable to perform all our experiments with normal B cells, it seems A20 is an acceptable replacement. Results from paper 1 and paper 2 suggest that A20 may have an enhanced capacity to stimulate Id-specific T cells with Id^+, compared to normal B cells. We investigated A20 B lymphoma cell surface marker expression in response to idiotypic stimulation (paper 1 and 2). However as we did not do this in parallel for normal B cells, it is difficult do say if the up or downregulation of cell surface markers was within the normal range.

IgD as an Id^+/anti-Id receptor on A20 (paper 1 and 2)

The A201.11 has endogenous membrane bound IgG2a (237), and no membrane bound IgM, IgA (238). We transfected A201.11 with Id^+ or anti-Id membrane bound IgD. This gave B lymphoma cells that expressed Id^+ or anti-Id membrane bound IgD with endogenous membrane bound IgG2a. We assume that endogenous membrane bound IgG2a did not effect our results greatly, as it has no specificity for our Id^+ or anti-Id Ig.

Naturally IgD is co-expressed with IgM on peripheral mature B cells. Would the presence of idiotype specific (Id^+/anti-Id) membrane bound IgM on our transfected A20 cells significantly change the results we obtained for antigen processing and presentation, and for apoptosis induction, in paper 1 and 2 respectively ?

IgD function is still somewhat enigmatic. The protein structure of IgD and IgM is similar. The same signaling Ig-α/Ig-β heterodimer is coupled to both membrane bound IgD (mIgD) and membrane bound IgM (mIgM). The mIgM and mIgD molecules have the same cytoplasmic tail consisting of only three amino acids (1). The cytoplasmic components of the mIgM/Iα-Ig-β and mIgD/Iα-Ig-β antigen receptor complexes should thus be identical.

Some reports demonstrate that early events in signal transduction are similar for mIgD and mIgM (239,240). However the downstream effects of this signaling may vary. In experiments with B lymphoma cells expressing mIgM and mIgD, cross-linking of mIgM induced growth arrest, whilst cross-linking of mIgD did not induce growth arrest (241). Also there are reports of tolerance-induction and apoptosis of B cells differing, depending on the presence or absence of mIgD. In experiments using transgenic mice with anti-TNP mIgM B cells, addition of TNP caused deletion of the self reactive B cells. Whilst in transgenic mice that carried an additional δ transgene, TNP addition did not cause deletion of the self-reactive B cells (242), implying that sIgD interfered with tolerance induction. In a mouse model for rheumatoid arthritis one transgenic mouse expressed self reactive B cells with mIgM and mIgD and another transgenic mouse expressed self reactive B cells with only mIgM. When exposed to antigen, B cells in mice with self reactive mIgM only B cells were deleted. Self reactive B cells in mice expressing mIgD and mIgM were resistant to deletion (243). This suggests that mIgD can increase the threshold for negative selection, by modulating signal strength. It appears that although structurally similar, the effect of signaling through mIgM or mIgD is different, at least in terms of growth inhibition and tolerance induction.

Even though our Id^+ A20 and anti-Id A20 cells only express Id^+- and anti-Id mIgD respectively (not Id^+ or anti-Id mIgM), they are indeed susceptible to apoptosis (paper 2). Given the above observations, the addition of Id^+ or anti-Id mIgM to our A20 cells might enhance apoptosis.

Different from the above experiments investigating the loss of mIgD, experiments have been performed where the consequence of mIgM loss was investigated. Here B cells expressing only mIgD were compared with normal mIgM/mIgD expressing B cells. These experiments demonstrated that mIgD largely can substitute for loss of mIgM. B cells with only mIgD supported normal B cell responses, including isotype class switching during immunization or infection (244). So it is possible that our Id^+- and anti-Id mIgD expressing A20 cells can use mIgD to substitute for mIgM functions. Thus, the effect of adding Id^+ mIgM to our Id^+ mIgD expressing A20 cells, and anti-Id mIgM to anti-Id mIgD expressing A20 cells, may not greatly effect the outcome in terms of apoptosis or antigen processing and presentation.

Purification of Abs (paper 1,2,3).

In paper 1 recombinant anti-IgD Ig with Id^+ peptide was isolated from supernatant of transfected cells and purified on a protein G column. The resulting recombinant Ig preparation would contain bovine Ig as well. This is a concern as bovine Ig could bind to FcR and influence results. The NIP controls were purified on a 4 hydroxy-3 nitrophenacetyl (NP) column, and should contain no bovine Ig.

Recombinant anti-IgD Igs with different antigenic T cell epitopes integrated, have been purified on both protein L and protein G columns. Protein L columns do not bind bovine Ig. Results with these recombinant Ig preparations were the same (not published). Thus bovine Ig contamination in recombinant anti-IgD preparations should not be a problem.

M315 (α, λ2) mAb, Id^+ Ig and isotype control M460 (α, κ) mAb, Id^- ctrl Ig, were isolated based on their capacity to bind DNP ligands (245). Thus, the isolated Ig should be bovine Ig free.

B cell activation signals *in vitro* (paper 3)

The activation state of both B cells and T cells is an important consideration for paper 3. As we wanted to determine the sensitivity of the Id-specific T cell - B cell collaboration, both B and T cells used should be naïve and non-activated.

All the mice we used were kept in a pathogen monitored environment at our animal facility. Both B and T cells should be naïve in the sense that they had not been exposed to antigen (except for Id-specific Th2). However as B cells in some experiments were purified with anti-CD19 beads, it could be argued that this induced B cell activation. If so, the B cell could have an enhanced capacity as an APC. It has been demonstrated that ligating IgM can induce upregulation of the B7 marker (246). B7 is associated with an enhanced B cell activation state (247).

CD19 is part of a signaling complex (248), and mobilizes intracellular calcium by mAb cross-linking of CD19 and co-receptors known from the BCR activation pathway (249). B cell antigen-specific activation is enhanced by the CD19/CD21/CD81 complex (250). However selective ligation of CD21 inhibits BCR mediated activation (251). Also selective ligation of CD19 can down-regulate signaling and inhibit proliferative responses

(252,253). It does not seem very likely that purification of B cells by using anti-CD19 beads would induce an enhanced activation state of the cells.

There were no great differences in Id-specific T cell proliferation responses induced by B cells either positively selected (CD19) or purified by negative depletion. Also if purification of B cells with anti-CD19 mAb induced an enhanced activation state of the B cells, we should have observed better Id-specific T cell responses for BALB/c B cells with Id^+ Ig.

Activation of naïve Id-specific T cells *in vitro* (paper3)

Naïve Id-specific T cells were used as CD8 depleted LN cultures from TCR TG BALB/c in some *in vitro* experiments. One could argue that these T cells could be activated by other APC than anti-Id B cells. However when such Id-specific T cell cultures were incubated with Id^+ Ig, but no anti-Id B cells, there was no Id-specific T cell proliferation (not shown).

Activation of naïve Id-specifc T cells in vivo (paper 3)

Adoptive transfers with naïve Id-specific T cells, anti-Id B cells and Id^+ Ig resulted in Id-specific T cell proliferation. The level of T cell proliferation was absolutely dependent on anti-Id B cells being present. Our *in vitro* results suggest that the Id-specific T cell - B cell collaboration takes place in absence of other APC. However, *in vivo* in BALB/c recipients, we cannot rule out, that endogenous APCs to some degree could prime and activate Id^-specific T cells. This could be ruled out by transferring purified Id-specific naïve T cells and anti-Id B cells with Id^+ Ig to an immune-deficient mouse strain with another MHC class II haplotype than BALB/c (H-2d). In this way highly purified Id-specific T- and anti-Id B cells could be transferred into recipients lacking the required MHC class II for presentation of Id^+ to Id-specific T cells.

Sensitivities of ELISA (paper 3)

In paper 3 we wanted to determine a lower threshold for cells and Id^+ Ig that could induce an Id-specific T cell - B cell response. Our most sensitive read-out was ELISA, measuring isotype switched anti-Id Abs in serum. An unexpected result, was that IgG2b was the most dominant anti-Id Ab isotype as a result of anti-Id B cells, Id-specific T cells and Id^+Ig interacting *in vivo*. From this given interaction, T helper cell responses should be expected. Th1 and Th2 promote mainly B cell isotype switching to IgG2a and IgG1 respectively (254). Tfh largely promote B cell isotype switching to IgG1 (255) and Th17 promote B cell isotype switching to IgG2a and IgG3 (256). Thus, it is likely that our ELISA parameters are not optimal, resulting in different detection sensitivities for the different Ig isotypes. Possibly the "linked" Id-driven T cell – B cell interaction is relevant at even lower numbers of cells and Id^+ Ig than we have estimated based on ELISA readouts.

DISCUSSION

The theme for this thesis is Id-driven interactions of specific T- and B cells, either B - B or T - B. We propose that these interactions play a part in regulating idiotype networks. We show in a surrogate model that anti-Id B lymphoma cells are very efficient at inducing Id-specific Th cell functions in the presence of Id^+ Ig (paper1). We demonstrate that idiotype specific B - B interactions, could reduce the B cell repertoire, and thus idiotypic connectivity (paper 2). In paper 3 we present a physiological model for "linked" Id-driven T cell - B cell collaboration. We demonstrate *in vivo,* that this type of collaboration is highly sensitive for inducing anti-Id isotype switched Abs in response to non-adjuvanted Id^+ Ig.

BCR diversity

Clonally distributed BCRs have extremely diversified variable (V) regions due to random recombination of V(D)J gene segments, junctional diversity and somatic hypermutation (257). About 10^8 B cell lineage precursors are generated daily in murine bone marrow, resulting in $2x10^7$ immature membrane IgM expressing B cells (258). Given this high output of B cells with randomly generated BCR, self-reactive B cells should arise frequently (11). In fact monoclonal antibodies cloned from single purified B cells show that 55 to 75% of all antibodies expressed by early immature B cells display self-reactivity (259). Most of these are removed at checkpoints, some as immature B cells in the bone marrow, others as transitional/new emigrant B cells (259). However some self reactive B cells escape tolerance induction and are present in the periphery (20).

Idiotypes and the idiotypic network

As a consequence of BCR diversity, individual Ig molecules carry unique V region antigenic determinants that are called idiotopes (104-106). Idiotopes can be recognized by

other antibodies in the individual. This is illustrated by high idiotypic connectivity in neonates (260) and also in adults (261).

Based on several early experiments (see introduction "the idiotypic network") N.K Jerne postulated that the immune system functions as a network based on idiotypic interactions between lymphocytes expressing complementary BCRs (108). However T cells were not integrated into this model.

T cell tolerance to immunoglobulin V regions

It has been demonstrated that APCs process and present Ig V-region derived idiotypes on MHC class II molecules to Id-specific $CD4^+$ Th cells (199,200,262). Further, it has been demonstrated that B cells process endogenous BCR and present Id peptides on MHC class II (78,192,263).

If naïve T cells responded to all idiotypes, there would be uncontrollable autoimmunity, so this is obviously not the case. Several restrictions apply. There are limitations to the Id peptides that can be presented on MHC class II (201). The frequency of $CD4^+$ T cells with TCR able to recognize Id may be low, due to restricted TCR use (264). Also T_{regs} may have a dampening effect. Further, the diversity of Id peptides available for T cell recognition, is much lower than the diversity of idiotypes available to B cell recognition. This is so because T cells recognize sequence determinants while B cells recognize conformation determinants to which Ig H and L chain pairings contribute. Also T cells appear to be tolerant to germline Id, and only recognize either somatically mutated, or non-germline sequences (N region diversity) (200,265,266). Tolerance induction of Id-specific peripheral T cells may also arise in a concentration dependent way, as shown for myeloma protein in myeloma protein specific TCR transgenic mouse (267). Despite all these restrictions, expanded pools of idiotype specific T have been shown to exist in humans with diseases such as SLE (268), rheumatoid arthritis (269) and multiple sclerosis (270).

"non-linked" Id-driven T cell - B cell collaboration

B cells process endogenous BCR and present Id peptides on MHC class II (78,192,263). An experimental model with Id-specific Th cells from TCR transgenic mice

(202) and Id^+ B cells from BCR $\lambda 2^{315}$ transgenic mice (271) demonstrated that, Id^+, $\lambda 2^{315}$ expressing B cells, in absence of ordinary antigen, isotype switch and enter GC reactions, in the context of Id-specific Th cells (194). This T cell - B cell interaction has been termed "non-linked" Id-driven T cell - B cell collaboration (see introduction for details). Chronic T cell stimulation of B cells in the "non-linked" Id-driven T cell - B cell collaboration, can lead to B cell lymphoma development (195) and SLE (196).

"linked" Id-driven T cell - B cell collaboration

It has been demonstrated that B cells can present exogenous Ig Id in a MHC class II restricted fashion to Id-specific T cells (200). However, in this system spleen APCs were used, thus no anti-Id BCR was present. We show (paper 1 and 3) that using normal B cells with anti-Id BCR, Id^+ Ig is efficiently processed, and presented to Id-specific Th cells eliciting Id-specific Th cell and anti-Id B cell effector functions. This Id-driven T cell - B cell collaboration is "linked" in the sense that the BCR and the TCR recognize components from the same antigenic entity (Id of the Ig).

Tolerance induction to self-reactive idiotypes. Id^+ A20 and anti-Id A20 induce unidirectional apoptosis mediated by BCR/BCR interactions.

What would happen if an Id^+ B cell and an anti-Id B cell were to encounter? To answer this we generated B lymphoma cells with idiotypically complementary BCRs.

As a mechanism of tolerance induction, it has been demonstrated that high affinity BCR interactions with self-antigen induce apoptosis (272). In paper 2 we observed apoptosis for Id^+A20 and anti-Id A20 when interacting in a BCR/BCR specific manner. Also anti-Id A20, with a higher BCR density than Id^+ A20, was induced to undergo apoptosis when incubated with Id^+ Ig. By contrast, we could not observe any significant apoptosis for Id^+A20 incubated with anti-Id Ig. This is consistent with previous findings showing that BCR density (overall binding-capacity) influences the kinetics of apoptosis for B cell: B cells with high density of self reactive BCR are more prone to apoptosis than B cells expressing lower density of self-reactive BCR (273,274).

Previous reports show that BCR mediated binding to membrane bound self-antigen is more potent at inducing signaling than BCR binding to soluble self-antigen (205,275,276). This was supported by our findings. For the Id^+/anti-Id BCR/BCR interaction, we demonstrated that both cells are capable of undergoing apoptosis. However, overall, Id^+ A20, with comparatively lower BCR density, was more susceptible to apoptosis. An additional finding, was that only one of two interacting cells received a caspase 3 apoptotic signal. These observations are puzzling.

It is difficult to speculate how the above mentioned events arise. Experiments using B cells interacting by idiotype specific BCR/BCR interactions, have not been performed previously. Experiments have been done with Hen Egg Lysozyme (HEL)-specific B cells and HEL conjugated to an immobilized membrane, or to HEL expressing cells (48,277). These experiments show that when the HEL-specific BCR meets membrane bound HEL, HEL will be gathered into a defined cluster by the HEL-specific B cell spreading its membrane over the target membrane. This membrane spreading first generates several micro-clusters of BCR/antigen, and then contracts into a focal point gathering all the smaller clusters into one bigger cluster (48). Below a certain threshold of HEL antigen, there is no B cell membrane spreading and thus no signaling (48). In our case the conventional HEL antigen would be Id^+ BCR or anti-Id BCR. If antigen density, or BCR density in our case, is a determining factor, then there could be a skewing towards Id^+A20 spreading its membrane over anti-Id A20, and not the other way around. Anti-Id BCR could then be gathered into signaling micro-clusters, and subsequently into one larger cluster. Anti-Id would also be capable of initiating the membrane spreading, but this event would probably be less frequent, due to the decreased likelihood of anti-Id BCR sensing a Id^+ BCR dense membrane surface area.

The term trogocytosis, defines the intracellular transfer of plasma membrane between cells of the immune system (278). Recent reports have demonstrated that B lymphoma cells (279) and normal B cells (280) have the capacity to transfer membrane. It has been demonstrated that two normal B cells can efficiently transfer BCR between each other (280). It has also been proposed that capture of such membrane components from one cell to another, can confer sustained intracellular signaling for the cell that captures membrane components (hypothesized for T cells interacting with APCs (281)).

All taken together, I speculate, that in the majority of encounters, Id^+A20 will spread its membrane over anti-Id A20, triggered by the higher BCR density of anti-Id A20. Speculatively, due to this directional skewing of the membrane interaction, Id^+A20 could

trogocytose membrane from anti-Id A20 and internalize anti-Id BCR which could lead to sustained intracellular signaling for Id^+A20. This would finally send a caspase signal to Id^+A20 (the possible process is illustrated in Figure 11). We have observed trogocytosis-like cell morphology in our Id^+/anti-Id BCR/BCR interacting cells, see Figure11.

Following the possible explanation above, the remaining anti-Id A20, having lost BCR/membrane components, could have lost the potential to reach the BCR activation induced threshold level of Ca^{2+} needed for apoptosis (282). This could explain the unidirectional caspase activation signal.

Isolating cellular interactions that could demonstrate the speculative explanation given above is difficult, as the cells interact in clusters with different numbers of cells. However the fact that cells interact in single conjugates and larger clusters, does not exclude the above explanation.

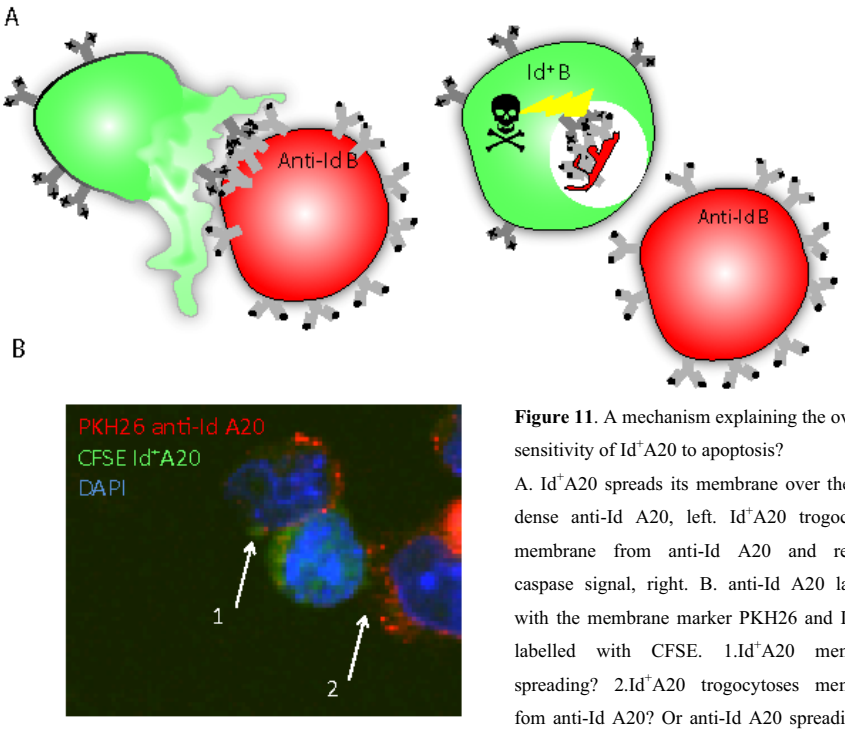


Figure 11. A mechanism explaining the overall sensitivity of Id^+A20 to apoptosis?
A. Id^+A20 spreads its membrane over the BCR dense anti-Id A20, left. Id^+A20 trogocytoses membrane from anti-Id A20 and receives caspase signal, right. B. anti-Id A20 labelled with the membrane marker PKH26 and Id^+A20 labelled with CFSE. 1.Id^+A20 membrane spreading? 2.Id^+A20 trogocytoses membrane fom anti-Id A20? Or anti-Id A20 spreading it's membrane over Id^+ A20?

Three known self-tolerance mechanisms regulate autoreactive B cells: apoptosis, receptor editing and anergy (272,275,283-285). These individual models predict that BCR ligation leads to vastly different outcomes for the B cell. Several factors can influence the outcome;

ligand affinity (205,275,276), expression of mIgM as B cells mature (286), or extrinsic factors delivered by the microenvironment (206). So hypothesizing an outcome for normal B cells engaged in idiotype specific BCR/BCR interactions is difficult. However it seems likely that the idiotype specific BCR/BCR interaction with ensuing apoptosis could illustrate a tolerance mechanism for ridding the body of self reactive B cells, in a selective manner. In our case the selective advantage is conferred by a higher BCR density. B cells in the periphery are highly dependent on the BCR for survival (287). So possibly the selective apoptosis we describe could serve to eliminate B cells with a lesser capacity to receive BCR survival signals.

Examples of idiotypic connectivity

Self reactive B cells, as discussed above are normally eliminated by tolerance mechanisms. However B cells that are self reactive to other Ig idiotypes, often seem to escape tolerance mechanisms. This is demonstrated by the high idiotypic connectivity in neonates (260), by the induction of anti-Id Abs in individuals immunized with monoclonal Ig (106) and by the presence of anti-idiotypic Abs towards pathogenic autoantibodies in healthy individuals (122,123).

It has even been demonstrated that immunization with Ig can potently elicit cascade reactions of idiotypes (all escaping tolerance), leading to Ig present in a sufficient concentration to kill tumors. Gangliosides are expressed on tumors (288,289). When non-small lung cancer patients were immunized with a murine anti-idiotypic mAb specific for an idiotypic mAb reacting with N-glycolyl containing-gangliosides, a tumoricidal effect was observed in patients (290). In a similar model a tumoricidal effect was obtained with mAb directed to p53 antigen in immunized mice. The immunized mice produced IgG antibodies to p53 and mounted a cytotoxic reaction to a tumor-line bearing mutated p53 (291).

In both experiments, tumoricidal IgGs resulted (126,291). This possibly means that the tumoricidal IgGs arise from a GC reaction (292). GC reactions could lead to idiotypes with highly mutated Ig V regions (292). B cells expressing such highly mutated self reactive Igs could escape tolerance mechanisms (293).

Lack of tolerance to B cells expressing self reactive non-germline idiotypes. Id^+ Ig M315 mAb and anti-Id Ab2-1.4 are non germline idiotypes.

Id^+ Ig mAb M315 used in our system, has the rare λ2 isotype (294). Also it has somatic mutations in this λ chain in position 94, 95, 96, conferring aa substitutions from Tyr, Ser, Thr to Phe, Arg, Asn (294-296). Anti-Id mAb Ab2-1.4 (297) is also heavily mutated leading to aa substitutions. Ab2-1.4 V_H has somatic mutations leading to 9 aa substitutions and additional N-region nucleotides leading to 3 additional aa (paper 3). When mice were hyper-immunized with Ab2-1.4 mAb, M315 like Abs were generated (298). This could suggest that an Ig V(D)J with a high degree of somatic mutations, N-region additions or imprecise joining of gene elements, could lead to rare idiotypic variants that would escape deletion. Lupus prone mice mice that are defient for Terminal deoxynucleotidyl transferase (TdT), which adds nontemplate coded nucleotides (N-region additions), have a decrease in autoantibodies (299). This suggests that N region nucleotide additions alone, could be enough to create tolerance escaping clones.

It has been suggested that whilst there is tolerance towards the abundant germline idiotypes, the rare e.g somatically mutated versions will be of such low concentration that they cannot induce tolerance in the same way (300). Could it be that such rare idiotypes would not have been presented to T cells, during the development of central tolerance and thus such specific T cells could be activated in the periphery. If this is the case, one could speculate that idiotypic connectivity is different depending on different B cell subsets.

A speculative model for idiotypic connectivity defined by B cell subsets

Follicular B2 cells constitute the majority of B cells in spleen (~70%) (22). These B cells depend on Tfh cells in order to become effector B cells. When they receive Tfh cell help, they can aquire somatic hypermutations in V genes, isotype switch and produce high affinity Abs (88). B-1 cells, either B-1a or B-1b constitute 2% and <1%, respectively of B cells spleen (301). B-1 cells mainly reside in peritoneal and pleural cavities (constituting 35-70% of cells) (22). B1-a cells have unmutated V regions, and express very few if any N-region substitutions in their CDR3 region (302). B1-b cells by contrast contain N-region additions in their CDR3 sequences (303). MZ B display N region diversity in CDR3, but at a lower level than for B2 follicular B cells, as well as displaying V gene somatic hypermutations (304,305). In contrast to the follicular B2 cells, B1 and MZ readily

proliferate in response to lipopolysaccaride (LPS), reviewed in (27). In this T-independent fashion, they have features of innate immunity (reviewed in (27)). B-1 cells can even spontaneously secrete IgM (306). Last but not least, B-1 cells and MZ B cells are enriched for self reactive clones that also can bind bacterial antigens (33,307). B1 cells produce low affinity IgM and act early on in the immune response, in comparison to B2 cells that act in the adaptive immune response and can generate high reactive clones (301). In light of all this, cold it be that B cells, depending on their subset, have different idiotypic connecitivity?

Perhaps B-1a cells with a low threshold for T-independent activation and subsequent Ig secretion, expressing low affinity self Ig, have a web of self-idiotype reactive low affinity Igs. These Igs would have germline encoded idiotypes of such abundance that T cells would be tolerized to them. A similar, but reduced idiotypic connectivity could exist for MZ B cells, also able to secrete Ig T-independently. MZ B cells would have a higher degree of idiotypic diversity, provided by N-region substitutions and somatic mutations. Given this larger idiotype diversity, clones with higher affinity for self idiotypes, could arise. Of interest, MZ B cells show pronounced IgM induced apoptosis, which is not present for B-1 cells, and to a lesser extent for follicular B2 cells (31,308). This could illustrate a need to get rid of possible self reactive clones of high affinity. Speculatively, for B-1 cells and MZ B cells, low affinity IgM interactions would supply a network of idiotype connections.

Finally we have the bulk of the splenic B cells, follicular B2 cells. Follicular B2 cells require Tfh cell help to expand in response to antigen. In the absence of adequate Tfh cell help during B cell priming, follicular B2 cells arrest in the T-cell zone after binding antigen and undergo apoptosis rather than differentiation into GC B cells or antibody-forming plasma cells (309,310). Thus almost all follicular B2 cells reacting to germline Id would die as Tfh cells would be tolerized to these abundant self-idiotypes. However it has been demonstrated that MZ B cells can shuttle between the marginal zone and the B cell follicle containing follicular B2 cells (311). So speculatively, a MZ B cell expressing an N-region substituted, somatically hypermutated Ig, could be recognized by a follicular B2 cell. The latter cell could present peptides to an Id-specific Tfh cell. This Id-specific Tfh cell would not have encountered such a non-germline idiotype during development of central tolerance and thus be responsive. Subsequently, the Id-presenting B cell would become an effector B cell secreting anti-idiotypic antibodies. Under normal circumstances this would probably not happen, as there would have to be a sufficient concentration of

non-germline Id present to initate the “linked” Id-driven T cell- B cell collaboration described. However if e.g LPS provided a help signal for the initiating MZ B cell, this could increase the concentration of the N-region substituted, somatically hypermutated Ig to a sufficient concentration to initiate the “linked” Id-driven T cell- B cell collaboration. It has been suggested that there is a link between bacterial infection and SLE (312). Maybe the expansion of non-germline Id expressing MZ B cells under the influence of bacterial derived LPS could trigger an expansion of high affinity anti-idiotypic B cells capable of binding DNA. If the infected individual had a reduced capacity to clear bacteria, e.g a complement deficieny, this could increase the level of the activating LPS.

If the follicular B2 cell producing anti-idiotypic Ig to MZ B cell Ig, receives sufficient Id-specific Thf help, then the follicular B2 cell could produce somatically hypermutated Ig. This Ig could be presented to another Id-specific Tfh cell by “non-linked” T cell- B cell collaboration. Thus there could be expansion of several high affinity Ig producing follicular B2 cells. It has been shown that chronic “non-linked” Id-driven T cell-B cell collaboration can lead to SLE with the production of the disease related autoantibodies (196). Such antibodies are somatically hypermutated (293).It has also been demonstrated that there is an explosion of autoantibodies in SLE patients, many of which are correlated with diseae activity (313). How “linked” and “non-linked” Id-driven T cell-B cell collaboration could work in concert will be discussed further on. See Figure11 for an illustration of the speculative network of idiotypic interactions, depending on B cell subsets.

The mechanism for how non-germline idiotype producing B cells are expanded by help from non-tolerized idiotype-specific Tfh cells, could explain how the introduction of exogenous idiotypes, readily can initiate idiotype cascades and result in high concentrations of Ig capable of killing tumors (314).

In addition to Ig V genes being somatically hypermutated during the GC reaction (315), non-Ig genes can also be mutated in this process (316). Mutations in the CD95 gene, implicated in negative selection of GC B cells, frequently arise in parallel with somatic hypermutation, and may lower the threshold for self reactive clones arising (317).

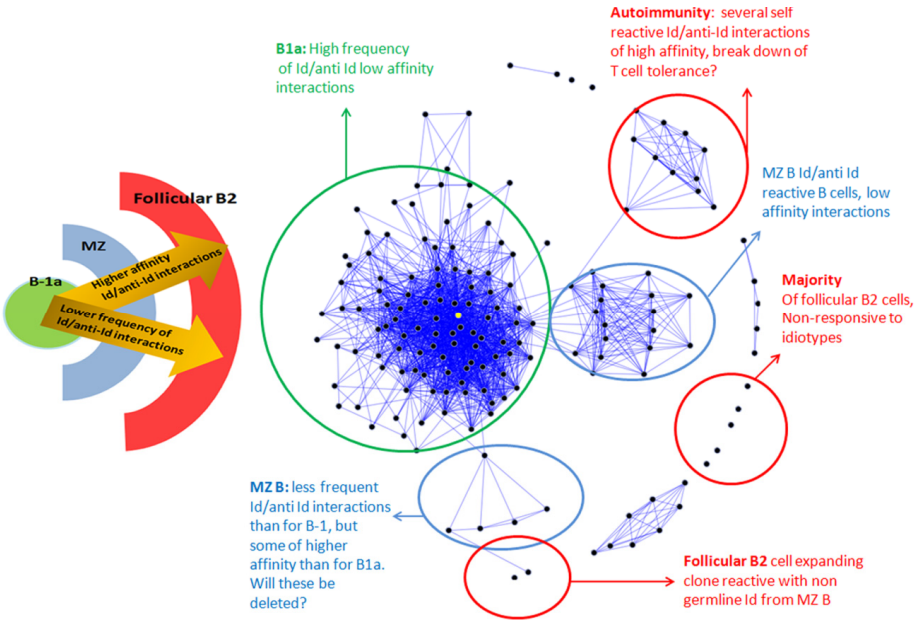


Figure 11. Possible distribution of Id/anti-Id interactions between different B cell subsets. Dots signify cells. Lines signify an Id/anti-Id interaction. Dots do not represent a realistic distribution of cells in each B cell subset population. Possibly B1a cells (center, green) have a high degree of idiotypic low affinity connectivity. The number of Id/anti-Id interactions could be decreased for MZ B cells (middle, blue), and further reduced for B2 follicular B cells (outer zone, red). The affinity of the interactions could be reversly proportionate to the frequency of interactions, with B1a cell Id/anti-Id interactions being high frequent but low affinity and follicular B2 cell Id/anti-Id interactions being very low frequent, but of high affinity. However during autoimmune disease there could be a break-down of Id-specific Thf cell tolerance, resulting in the generation of several high affinity Ig idiotypes.

Naïve Id-specific T and naïve anti-Id B are sufficient to respond to Id^+Ig

Data in paper 3 demonstrates that naïve anti-Id B cells and naïve Id-specific T cells are sufficient in the presence of Id^+ Ig to initiate anti-Id B cell responses. Classically B cells have not been regarded as very efficient APCs. B cell deficient mice have a normal capability of T cell priming (318). However this was shown in lack of a BCR specific for antigen. Tetanus toxoid specific B cells have been shown to trigger T cell proliferation at 10^4 x lower Ag concentration than non-specific B cells (319). Also it has been shown that specific B cells are required for systemic T cell priming at low antigen concentrations (320).

Previous findings show that naïve B cells require an activation signal in order to activate naïve T cells (321). Experiments using HEL specific naïve T and B cells show that BCR mediated stimulation, activating the BCR complex (up-regulation of B7 co-stimulatory molecule), is a sufficient activation signal for rendering the B cell capable of stimulating T cells *in vitro* (322). We here show that anti-Id B lymphoma cells exhibit $x10^4$ capacity to stimulate Id-specific T cells in *vitro* (paper 1). *In vivo* Id^+Ig binding to anti-Id BCR induces a highly efficient stimulation of naïve Id-specific T cells (~130 ng/ml Id^+ Ig).

If naïve T cells and naïve B cells with small amounts of Id^+ Ig are enough to induce anti-Idiotypic responses, this could lower the threshold for self-reactive anti-Id idiotypes escaping tolerance.

Combining the "Non-linked" and "Linked" Id-driven T cell - B cell collaboration in one model

The "linked" Id-driven T cell - B cell collaboration described in paper 1 and 3 needs to be integrated into one model with "non-linked" Id-driven T cell - B cell collaboration (see Figure7 paper 1 and extensive discussion paper 1). How "linked" and "non-linked" Id-driven T cell- B cell collaboration can work in concert was briefly discussed above in "A speculative model for idiotypic connectivity defined by B cell subsets". However the sum of interactions involved could be more complex than previously discussed.

An Id^+ B cell (to the left in Figure 12) can process it's endogenous monoclonal BCR and presents peptides on MHC class II to Id-specific T cells *(i)* (see Fig. 12 for symbols). Also BCR can be ligated by polyclonal anti-Id *(ii)* and *iii)*, resulting in presentation of Id-peptides derived from endogenous Ig to several Id-specific T cells (T_2, T_3). This also applies to an anti-Id B cell (to the right in Figure 12). Thus the complementary Id^+ B cell and anti-Id B cell are probably regulated by partly overlapping sets of Id-specific T cells. This is a simplification, and would be influenced by many factors: 1) different IgG isotypes would have different ability to bind FcRs, some would bind FcRIIb1 and deliver negative signals to the B cell (230), 2) different T cell subsets would have different effector functions (Th1, Th2, Th17, Tfh, T_{reg}), 3) MHC class I restricted Id-specific $CD8^+$ T cells could kill B cells, 4) $CD4^+$ T cells seem tolerant to

abundant germline-encoded Id-peptides (200,265,323). However the last point could be circumvented by the introduction of somatic hypermutations/N-region substitutions in Ig Id-peptides being presented to T cells, as previously discussed. It could be argued that "linked" or "non-linked" Id-driven T cell B cell collaboration would create novel Ig V region mutations, destroying the T cell recognition of a given Id-peptide. However such mutated B cells could become subject to a novel sets of Id-specific T cells.

The picture that emerges for Id-driven T cell- B cell collaboration is complex. However such interactions could explain findings such as 1) neutralizing anti-idiotypic antibodies against autoantibodies to blood group antigens (324), 2) the large expansion of multiple self reactive Ig clones related to disease activity during (325,326) and prior to (327) autoimmune diseases, 3) somatic hypermutations of Ig V genes driven by the Id specific T cells as a cause of disease specific autoantibodies in SLE (293), 4) lymphoma development, as chronic Id-specific Tfh cell help with subsequent somatic hypermutations of Ig V genes, could lead to mutations in proto-oncogenes (195,328), promoting lymphoma development.

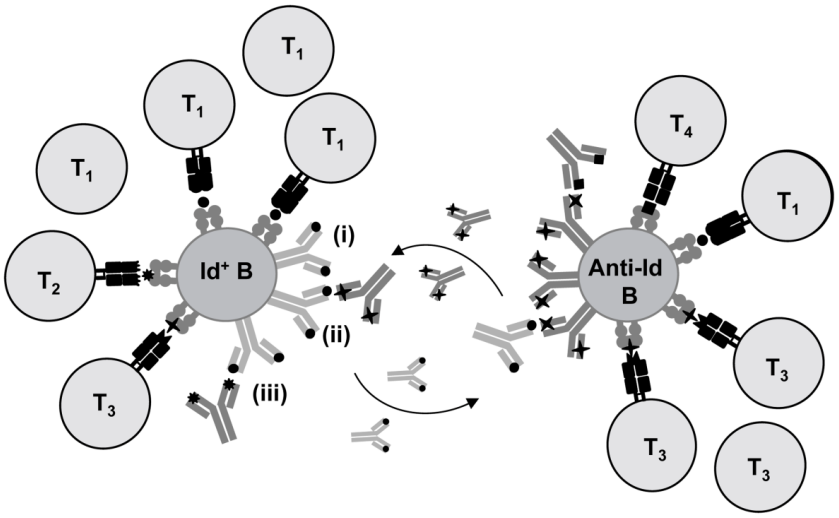


Figure 12. A revised idiotypic network model

BCR/BCR mediated apoptosis as shown in paper 2 would necessitate close proximity of B cells, and not necessarily exclude the interactions shown in Figure 12. B cell -T cell pairing may be favored over B cell-B cell pairings. Also soluble Ig may act with B cells (Id^+ Ig/anti-Id BCR and/or Id^+ BCR/anti-Id Ig) in such an extent that the two B cells cannot interact in a BCR/BCR mediated fashion.

FUTURE PERSPECTIVES

"Linked" Id driven T cell - B cell collaboration and lymphoma

As shown for "non-linked" Id-driven T cell - B cell collaboration, chronic Id-specific T-cell stimulation of Id^+ B cells induces B-cell lymphoma (195). If supplied with chronic stimulatory signal, through BCR (through the Id^+ Ig/anti-Id BCR interaction) and through Id-specific T cell help, would we observe the same for "linked" Id-driven T cell - B cell collaboration? It has been shown that BCR signal is instrumental in maintaining B cell lymphomas (329,330), thus it may well be that the proposed type of chronic stimulation could induce lymphoma development.

"Linked" Id driven T cell - B cell collaboration in tumor prevention

It has been demonstrated that Id-specific $CD4^+$ T cells can confer tumor resistance to MOPC315 (218). Given the excellent capacity of anti-Id B cells as APC for Id-specific T cells in the context of Id^+ Ig, would the presence of these B cells enhance the tumoricidal effect?

Various forms of Id as a BCR ligand, and the effect on "linked" Id-driven T cell – B cell collaboration

We are currently engaged in efforts to generate anti-Id, Ab2-1.4 specific T cells. Also a KI mouse for the HC of the Id^+ mAb M315 is being generated. A cross between this mouse and the $\lambda 2^{315}$ B cell transgenic mouse (271), would result in B cells expressing HC and LC of Id^+ mAb M315. The BCR of this Id^+ B cell could be ligated by the hapten dinitrophenol (DNP) for which M315 is specific. DNP could be conjugated at different ratios to small molecules (e.g. amino acids), macromolecules (proteins, carbohydrates), and even cells. Thus, a panel of antigens ranging from from monovalent to polyvalent could be easily generated. In addition to ligation with hapten, an M315-based Id^+ BCR could be ligated by the anti-idiotypic mAb Ab2-1.4. This antigen comes in many forms since our group has already expressed V-regions of anti-Id mAb with *i)* H-chains of various Ig subclasses (IgM, IgG1, IgG2a, IgG2b, IgG3), *ii)* as BCR on B lymphomas (paper1) and (*iii*) in double KI B cells (paper 3). Moreover, Fab fragments of Ab2-1.4

could be used as monovalent antigen. In summary, a range of haptenated antigens and protein ligands of various valency are available. These could be used to experimentally vary the degree of ligation and cross-linking of the Id^+ BCR, signaling of the B cell and presentation to anti-Id, Ab2-1.4 specific T cells. This could also be done with anti-Id B cells and Id-specific T cells, with different forms of Id^+ ligand.

Idiotypic B cell - B cell interactions, a physiological model

Obtaining the Id^+, M315 VH KI mouse would enable us to investigate the BCR/BCR complementary interaction of idiotypically paried B cells in a physiological manner. This is not trivial, since the B cells also express secretory Ig. However, labeling cells and using intravital multiphoton microscopy, we could perform adoptive transfers with different kintetics and study the outcome.

A revised network model

If we obtain anti-Id, Ab2-1.4 specific T cells and a VH 315 KI, we could investigate "linked" and "non-linked" Id-driven T cell - B cell collaboration simultaneously in one physiological model. We would have a model corresponding to that depicted in Figure12. Labeling cells and doing adoptive transfers with varying kinetics into recipient mice for intravital multiphoton microscopy could provide interesting data.

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