2006

Immunologic Memory to Polysaccharide Antigens

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A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

by

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June 2006

ABSTRACT

Immunologic memory is the ability of the immune system to generate an enhanced antibody response against previously encountered antigens. The clonal selection theory of acquired immunity attributes immunologic memory to the formation of an expanded compartment of quiescent antigen-specific memory B cells that are capable of rapid activation upon secondary antigen encounter. Whereas protein antigens are known to elicit memory B cells, it is not known whether polysaccharide antigens do.

Here we report that polysaccharide antigens elicit memory B cells that are phenotypically distinct from those elicited by protein antigens. We show that antigen affinity of the B cell receptor regulates the development of memory B cells and that antigen-specific IgG antibodies suppress recall responses against polysaccharides via a mechanism that does not depend on epitope masking or involve known Fcγ receptors.

ACKNOWLEDGMENTS

These studies were performed in the Laboratory of Molecular Immunology under the guidance of Dr. Michel Nussenzweig. I would like to thank the members of the Nussenzweig Lab, especially Tien-An Shih for teaching me basic techniques of immunologic research, Nikos Yannoutsos and Tom Eisenreich for help with generating BAC transgenic mice, Klara Velinzon for assistance with cell sort, Revati Masilamani and Mila Jankovic for informal exchange of ideas and suggestions for my project. I am indebted to Eric Meffre and Donal O'Carroll, who kindly provided mouse strains they had generated, and to Inês Crisóstomo, who assisted me with *S. pneumoniae* cultures.

I thank Drs. Ralph Steinman, Alexander Tarakhovsky, and Ian MacLennan for serving on my thesis committee. But most of all, I am grateful to my graduate advisor, Dr. Michel Nussenzweig, who warmly accepted me to his lab and, while giving me the freedom I needed to pursue my ideas, provided careful guidance and influenced my scientific development during the past five years.

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INTRODUCTION

Vaccination and Theories of Acquired Immunity

It has been known since ancient times that humans who recover from an infectious disease, such as smallpox, are spared when the epidemics returns. This resistance to reinfection was termed *immunity* from the Latin word *immunitas*, which originally meant the exemption from service to the state. In the early 18th century the practice of variolation became popular in England as a prophylactic measure against smallpox. It involved inoculation of a healthy individual with a virus from smallpox pustules of an infected person to elicit a less severe disease. At the end of the 18th century Edward Jenner introduced vaccination, a safer method of smallpox prevention by inoculating humans with *vaccinia,* a virus derived from cowpox pustules. A century later Louis Pasteur made a similar discovery that chickens that had recovered from mild cholera induced by an attenuated strain were resistant to subsequent challenge with a more virulent strain. This generalization of the Jennerian principle laid the foundation for the science of Immunology.

At the end of the 19th century, Emil von Behring and Shibasaburo Kitasato reported that animals immunized with diphtheria or tetanus toxin produced some substance in their blood (named anti-toxin) that could neutralize the toxin and prevent the disease. As a practical application of this finding, anti-sera obtained from experimental animals were used to cure diphtheria and tetanus in humans. Thus, anti-toxin (later renamed antibody) became one of the earliest recognized agents of acquired immunity against pathogenic antigens.

In the first half of the 20th century various theories were proposed to account for antibody formation, the earliest being the side-chain theory of Paul Ehrlich. He argued that bacterial toxins bind to complementary chemical patterns (side-chains) that are normally responsible for some metabolic function in the cell. To overcome the metabolic block, cells shed off and then regenerate their side-chains, which accumulate in the bloodstream. It soon became apparent that antibodies could form not only against a handful of bacterial toxins but virtually against any natural or artificial antigen, except self-antigens, and it started to appear unlikely that such an enormous repertoire of complementary side-chains could exist, as the theory predicted.

The side-chain theory was succeeded by the direct template theory, which attempted to explain antibody formation without postulating the enormous pre-existing diversity of recognition patterns. According to this theory, an antigen itself determines antibody specificity by entering a cell and serving as a template for antibody folding. This theory had to be abandoned after the discovery that it is the primary sequence of a protein that specifies its folding pattern. To accommodate this finding and to account for the persistence of antibody production, the indirect-template theory was proposed. According to this theory, a "genocopy" of an antigen incorporates into the genome of a cell, which allows it to specify the folding pattern of the antibody and to direct its production in descendant cells that have inherited the "genocopy".

A novel way of thinking about acquired immunity was inspired by Jerne's natural selection theory. Jerne proposed that immunoglobulins normally present in the serum carry reactive sites to any antigenic determinant, except those found in the body. An external antigen entering the body is responsible for selective carrying of a particular

immunoglobulin to the system of cells that can produce the same type of immunoglobulin in large quantities. Jerne's natural selection theory served as a precursor for Burnet's clonal selection theory, which postulated the cellular basis of humoral immunity. The hallmarks of the clonal selection theory are antigen-independent diversity achieved by means of somatic randomization and antigen-dependent selection resulting in preferential expansion of clones of a particular antigen specificity (Burnet, 1959). The main predictions of the theory are:

1) existence of a diverse repertoire of lymphocytic cells each carrying antigen-binding sites of a single specificity;
2) establishment of self-tolerance due to elimination of cells carrying self-reactive sites; and
3) clonal selection achieved by antigen-driven expansion of cells carrying antigen-specific sites with further selection of mutants with increased antigen affinity.

Subsequent studies in the second half of the 20th century have validated the clonal selection theory and elucidated cellular and molecular mechanisms responsible for the postulated features of the immune system.

B Cell Activation by T-Independent Type II Antigens

B lymphocytes (B cells) carry a membrane-bound form of antibody of a single antigen specificity called the B cell receptor (BCR), which consists of a pair of heavy and light chains. During the development in the bone marrow, B cell precursors undergo a stepwise assembly of their BCRs to generate the peripheral pool of mature B cells with a diverse repertoire of antigen specificities but limited self-reactivity. Upon antigen contact, mature B cells undergo activation and give rise to antibody-secreting plasma cells and quiescent memory B cells.

Characteristics of T-I type II antigens

Unlike soluble protein antigens, which are not capable of inducing antibody responses in athymic (nude) mice, some antigens are immunogenic in these animals and have been therefore called thymus-independent (T-I). They are further divided into type I and type II, according to their ability or inability to elicit antibody responses in CBA/N (Xid) mice (Mond *et al.*, 1995). T-I type I antigens include products of bacterial or viral origin, such as LPS, CpG, poly-IC, *etc.*, which activate B cells polyclonally via Toll-like receptors. On the other hand, T-I type II antigens such as polysaccharides and polymeric proteins engage BCRs and thus induce antigen-specific B cell responses. Typical T-I type II antigens include negatively charged or neutral capsular polysaccharides produced by some bacteria, such as *S. pneumoniae*, as well as synthetic haptenated polysaccharides, such as 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll.

The characteristics of T-I type II antigens are large molecular weight, high epitope valence (Dintzis *et al.*, 1989), and poor degradability *in vivo* (Britton *et al.*, 1968;

Sela *et al.*, 1972). Radioactive labeling revealed that many polysaccharides are retained in the liver and the spleen with an average half-life of more than 30 days (63 days for Ficoll) in mice (Humphrey, 1981).

Neutral and negatively charged polysaccharides do not bind to MHC class II molecules (Harding *et al.*, 1991). However, zwitterionic polysaccharides, synthesized by *B. fragilis* and *S. aureus,* can be processed to low molecular weight carbohydrates by a nitric oxide-mediated mechanism and due to their ability to form α-helixes can be loaded on MHC class II molecules and stimulate proliferation of $CD4^+$ T cells (Kalka-Moll *et al.*, 2002; Cobb *et al.*, 2004).

BCR signaling

By systematically varying the number and spacing of dinitrophenol (DNP) haptens on polyacrylamide (a T-I type II carrier), Dintzis *et al.* found that immunogenic preparations had a threshold number (of approximately 20 or more) of appropriately spaced (5-10 nm apart) repeating epitopes. Molecules with less than this number of haptens were not immunogenic at any dose (Dintzis *et al.*, 1976; Dintzis *et al.*, 1983). These studies, extended to other T-I type II carriers such as dextran and Ficoll (Dintzis *et al.*, 1989), suggested that multiple BCRs must be aggregated on the B cell surface by a T-I type II antigen to elicit B cell activation.

BCR crosslinking induces a complex signaling cascade. As the cytoplasmic domains of the heavy chains of the BCR are very short, BCR signaling is propagated through the cytoplasmic domains of non-covalently associated Igα and Igβ subunits, both possessing immunoreceptor tyrosine-based activation motifs (ITAMs) (Flaswinkel and

Reth, 1994). Aggregation of BCR complexes upon antigen binding causes their translocation to lipid rafts in the plasma membrane and facilitates tyrosine phosphorylation of ITAMs of Igα/Igβ by a *src*-family protein tyrosine kinase (PTK) Lyn and possibly other PTKs enriched in these microdomains (Cheng *et al.*, 1999). Phosphorylated ITAMs of Igα/Igβ then recruit and activate a cytosolic PTK Syk (Rowley *et al.*, 1995). This initial stage of BCR triggering is modulated by Csk, which phosphorylates the C-terminal inhibitory tyrosine of *src*-family kinases (Hata *et al.*, 1994). Acting in opposition to Csk is a transmembrane tyrosine phosphatase CD45 (B220), which dephosphorylates the inhibitory tyrosine of *src*-family kinases (Pao *et al.*, 1997) to ensure that they are in a state responsive to activation by BCR aggregation. Thus, the balance between the Csk and B220 activities regulates B cell responsiveness to BCR triggering.

Proper BCR signal transduction requires ordered activation of PTKs Lyn, Syk, and Btk. A crucial requirement for Btk function is its recruitment to the plasma membrane via the binding of its PH domain to PIP_3 generated by PI-3K phosphorylation of a plasma membrane lipid PIP_2 (Gold *et al.*, 1992; Saito *et al.*, 2001). Although a defect in the Btk's PH domain, such as the one found in Xid mice, does not abolish B cell activation and proliferation, it renders B cells activated by T-I type II antigens susceptible to apoptosis (Vinuesa *et al.*, 2001). Btk defects in humans lead to X-linked agammaglobulinemia (XLA) (Thomas *et al.*, 1993).

Activation of Syk and Btk leads to BLNK-dependent recruitment and activation of PLCγ2 (Fu *et al.*, 1998), which can cleave PIP_2 into secondary messengers, IP_3 and DAG. IP_3 generation stimulates increased Ca^{2+} influx into the cytoplasm leading to

activation of Ca^{2+}-dependent transcription factors (Dolmetsch *et al.*, 1997). DAG is required for activation of PKCβ, which is essential for phosphorylation of IKKα and subsequent activation of NF-κB (Saijo *et al.*, 2002). As is the case with Btk, activation of PKCβ is crucial for T-I type II antibody responses (Leitges *et al.*, 1996).

CD22 and FcγRIIB are known negative regulators of BCR signaling (Lankester *et al.*, 1995; Ono *et al.*, 1996). CD22 regulates Ca^{2+} efflux by plasma membrane calcium-ATPase-4 (PMCA-4) in SHP-1 dependent manner (Chen *et al.*, 2004). FcγRIIB-mediated inhibition is dependent on the inositol phosphatase SHIP, which dephosphorylates PIP_3 and thus reduces the levels of membrane-associated Btk (Ono *et al.*, 1996; Bolland *et al.*, 1998). Internalization of the BCR complex might also be a potent regulator of BCR signaling, since impaired BCR internalization in B cells with a mutated $Ig\beta^{Y2A}$ chain correlates with augmented T-I type II antibody responses *in vivo* (Gazumyan *et al.*, in press).

T-Independent Type II Antibody Responses

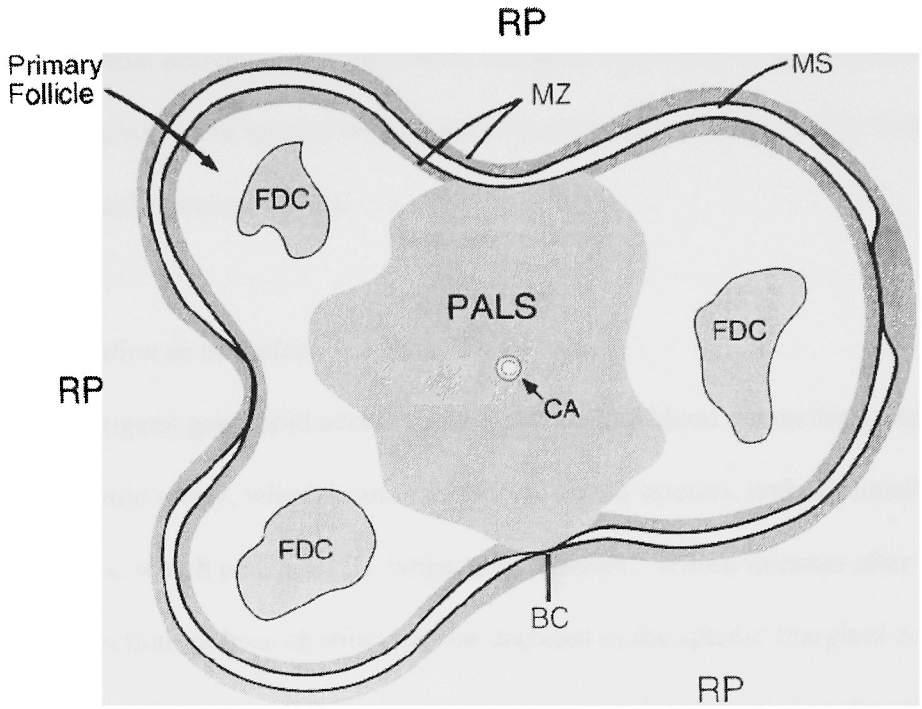
Splenic architecture and subpopulations of peripheral B cells

Immune responses take place in secondary lymphoid organs, such as the spleen, lymph nodes, and tissues associated with mucosal and gastro-intestinal surfaces. The spleen is the largest lymphoid organ containing 25% of mature lymphocytes. It is organized into two major areas: the red and the white pulp. The red pulp, the site of elimination of aged erythrocytes, also contains macrophages and plasma cells. The white pulp compartment contains mature B and T lymphocytes segregated from each other. A central T-cell rich zone, called periarteriolar lymphoid sheath (PALS), is surrounded by B cell-rich primary follicles, each containing a network of follicular dendritic cells (FDCs). The marginal sinus, lined with metallophilic macrophages, separates the white pulp from the red pulp, which are connected via the bridging channels containing $CD11c^{high}$ $DEC205^{-}$ dendritic cells (DCs) (Figure 1; reviewed in Fu and Chaplin, 1999).

B cells are organized into two compartments in the white pulp: primary follicles and the marginal zone (MZ). MZ B cells comprise about 10-15% of splenic B cells and are located on both sides of the marginal sinus. The MZ B cell compartment appears late in ontogeny and is indispensable for T-I type II immune responses. Factors responsible for cell adhesion are required for its formation, since interference with integrins, such as LFA-1 and its ligands ICAM-1 and VCAM-1, prevents B cell retention in the marginal zone (Lu and Cyster, 2002). Disruption of Pyk-2 kinase, a downstream component of integrin signaling, also results in the loss of the MZ B cell compartment (Guinamard *et al.*, 2000).

Figure 1. Structure of the white pulp nodule in the spleen.

Surrounding the central arteriole (CA) is a T cell-rich area called periarteriolar lymphoid sheath (PALS). Adjacent to PALS are B cell-rich primary follicles that include follicular dendritic cells (FDCs). The white pulp is separated from the red pulp (RP) by the bridging channels (BC) and the marginal sinus (MS), which is embedded in a layer of marginal zone (MZ) B cells. (Adapted from Fu and Chaplin, 1999).


RP
Primary
Follicle
MZ
MS
FDC
PALS
FDC
RP
CA
FDC
BC
RP

In addition to conventional B cells (follicular and MZ B cells), peripheral B cells include B-1 cells occupying primarily peritoneal and pleural cavities. B-1 cells form a self-renewing population that can get activated and differentiate to plasma cells without exposure to external antigens. B-1 cells have a limited repertoire of specificities and are thought to be the source of natural weakly auto-reactive antibodies normally found in the serum (Martin and Kearney, 2001).

Antigen localization in the spleen

Blood-borne antigens gain rapid access to the spleen. The blood enters the spleen through the splenic artery, which branches into trabecular arteries, and ultimately into central arterioles, which penetrate the white pulp nodules. Within minutes after intravenous injection, polysaccharides can be detected in the splenic marginal zone (Eertwegh *et al.*, 1992). Blood-borne bacteria are captured and carried to the splenic marginal zone by neutrophils and blood DCs (Balázs *et al.*, 2002).

Polysaccharide antigens are retained both in B cell follicles on FDCs and in extrafollicular regions on macrophages. The majority of the polysaccharide load is associated with the latter and is normally limited on the former. Follicular localization of polysaccharide antigens is dependent on complement and can be abrogated by cobra venom factor (CVF)-induced complement depletion. In contrast, macrophage depletion results in loss of polysaccharide accumulation in the marginal zone and the red pulp with concomitant increase in follicular localization (Eertwegh *et al.*, 1992).

GC reaction and extrafollicular response to T-I type II antigens

B cells that have been triggered by antigen initiate the germinal center (GC) reaction within B cell follicles or proliferate in extrafollicular foci, such as the bridging channels and the red pulp. Although the GC reaction is usually associated with T-D responses, GCs induced by T-I type II antigens have also been described, originally for α(1→6)dextran (Wang *et al.*, 1994). In wild-type mice, polysaccharide-induced GCs do not contain T cells (Vinuesa *et al.*, 2000) and can form in T cell-deficient mice (Lentz and Manser, 2001). T-I GCs are short-lived and support very low levels of somatic mutation of immunoglobulin V regions (Toellner *et al.*, 2002). Their functional significance is unknown.

T-I type II responses support class switch recombination, primarily to IgG_3 isotype. The Cγ3 transcript can be detected as early as day 1 and IgG antibodies appear in the serum by day 6 after NP-Ficoll immunization (Vinuesa *et al.*, 1999). The expression of activation-induced cytidine deaminase (AID), an enzyme required for somatic hypermutation and class switch recombination (Muramatsu *et al.*, 2000), has been analyzed in human lymphoid tissues, where it has been detected both in GC blasts and in extrafollicular large proliferating B cells (Cattoretti *et al.*, 2006).

During the extrafollicular response, activated B cells proliferate as plasmablasts in physical proximity to $CD11c^{high}$ $DEC205^-$ DCs (Vinuesa *et al.*, 1999) and require BAFF/APRIL to sustain their survival (Balázs *et al.*, 2002). Plasmablasts exit the cell cycle and terminally differentiate to plasma cells, which increase antibody secretion. The exit from the cell cycle requires a CDK6 inhibitor $p18^{INK4c}$ (Tourigny *et al.*, 2002), whose expression increases in response to IL-6 (Morse *et al.*, 1997).

Molecular requirements for plasma cell development

Differentiation to plasma cells and maintenance of the plasma cell fate are under the control of a transcription factor Blimp-1 (Shapiro-Shelef *et al.*, 2003; Shapiro-Shelef *et al.*, 2005). Through direct and indirect interactions, Blimp-1 represses genes involved in maintaining B cell fate (e.g. *Pax-5*), proliferation (e.g. *c-myc* and *E2F1*), and the GC function (e.g. *AID, Stat6, DNA-PK, Ku70*, and *Ku80*) (reviewed in Shapiro-Shelef and Calame, 2005). By down-regulating a B cell transcription factor *Pax-5*, Blimp-1 causes down-regulation of Pax-5-dependent genes, such as *CD19*, *Igα*, *syk*, and *BLNK*, and up-regulation of genes repressed by Pax-5, such as *XBP-1*, which in turn induces the expression of a large set of genes whose products participate in the expansion of the endoplasmic reticulum (ER) and in protein trafficking (Shaffer *et al.*, 2004). To be translated into a functional protein, *XBP-1* mRNA must be processed by IRE1α, an ER membrane-associated enzyme activated by the unfolded protein response (UPR) (Calfon *et al.*, 2002; Iwakoshi *et al.*, 2003). The UPR regulates the protein folding capacity of the ER and results in the remodeling of the secretory pathway during the physiologic transition from a non-secretory to secretory cell type or during pharmacologically induced ER stress.

Blimp-1 expression can be directly repressed by Bcl-6 via a conserved Bcl-6 response element found in the intronic region of the *prdm-1* gene encoding Blimp-1 (Tunyaplin *et al.*, 2004). Although transcribed in naïve B cells, high levels of the Bcl-6 protein accumulate in GC B cells (Fukuda *et al.*, 1997), in which Blimp-1 expression is restricted to a minor subset of Bcl-6$^-$ cells (Angelin-Duclos *et al.*, 2000). In GC B cells,

Bcl-6 is co-expressed with its cofactor MTA3, which is required for Bcl-6 acetylation and efficient suppression of Blimp-1 (Fujita *et al.*, 2004).

IRF4 is another transcription factor necessary for plasma cell development. *Irf4*$^{-/-}$ mice lack serum immunoglobulin and fail to be activated and proliferate in response to LPS *ex vivo* (Mittrucker *et al.*, 1997). Ectopic expression of the IRF4 transcriptional antagonist MITF leads to the same defect, whereas *Mitf*$^{-/-}$ B cells express higher levels of IRF4 and spontaneously differentiate into plasma cells (Lin *et al.*, 2004). Thus, IRF4 might be required earlier than Blimp-1 in the sequence of events leading to plasma cell production.

Plasma cell survival and apoptosis

Terminally differentiated plasma cells reside both in the splenic red pulp and in the bone marrow. The longevity of plasma cells had been debated. The lifespan of the majority of plasma cells in the lymph nodes and the spleen was estimated to be less than 3 days; however, plasma cells residing in the bone marrow and a small fraction of splenic plasma cells had a lifespan in excess of 3 weeks (Ho *et al.*, 1986) and some could be detected by autoradiography through the 6th month time point (Miller, 1964). Recently, the half-life of lymphocytic choriomeningitis virus (LCMV)-specific plasma cells in LCMV-infected mice was estimated to be approximately 170 days in the spleen and 90 days in the bone marrow (Slifka *et al.*, 1998). However, these studies focused on the plasma cell compartment established two weeks after the infection and did not rule out that plasma cells formed early during the immune response to the virus were short-lived.

Factors that determine the lifespan of plasma cells are not completely understood. Early plasma cell death is attributed to the finite capacity of the spleen to sustain them. No more than 20-100 plasma cells per mm^2 of spleen sections could be detected beyond day 7 after T-D or T-I type II immunization, irrespective of the number of B cells recruited into the response or the number of plasma cells produced before day 7 (Sze *et al.*, 2000). Plasma cells express death receptors DR4 and DR5 together with their ligand TRAIL and, unlike naïve or activated B cells, are sensitive to TRAIL-induced apoptosis *ex vivo* (Ursini-Siegel *et al.*, 2002).

Feedback Regulation of Antibody Responses

Immune complexes (ICs) modulate B cell responses by co-ligating the BCR and the inhibitory Fc receptor FcγRIIB on B cells. Fc receptors are also present on non-lymphoid cells, such as macrophages, DCs, and FDCs, thereby affecting antibody responses by regulating antigen localization and persistence.

Fc receptors

Antibody responses are modulated through the action of activating and inhibitory receptors. Fcγ receptors, ligated by Fc portions of IgG molecules, belong to both categories. Similar to Igα and Igβ, some activating Fcγ receptors contain ITAMs in their intracellular domains (e.g. human FcγRIIA) or are associated with the ITAM-containing FcRγ chain (e.g. human and murine FcγRI and FcγRIII) (Ravetch and Bolland, 2001).

Inhibitory receptors contain cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The only known inhibitory Fcγ receptor is FcγRIIB (Muta *et al.*, 1994). Targeted disruption of FcγRIIB results in disregulation of the immune system leading to augmented anaphylactic responses and spontaneous strain-dependent autoimmune phenotype (Takai *et al.*, 1996). Similarly, human patients with polymorphisms in the FcγRIIB promoter that lead to lower levels of FcγRIIB expression are prone to autoimmunity (Su *et al.*, 2004). IgG-containing ICs are crucial for the development of autoimmune symptoms, and FcγRIIB disruption leads to preferential augmentation of IgG titers, evident in the B6.56R anti-DNA BCR murine model (Fukuyama *et al.*, 2005).

In addition to its role in inhibitory signaling and modulation of autoimmunity, FcγRIIB is involved in trapping ICs on the surface of non-phagocytic FDCs and in the recycling of endocytosed ICs by phagocytic antigen-presenting cells, such as DCs. Unlike activating Fcγ receptors, FcγRIIB accesses a non-degradative vesicular compartment that recycles to the cell surface and preserves the associated antigen in its native form (Bergtold *et al.*, 2005).

B cells express FcγRIIB but not any known activating Fcγ receptors. A few Fc receptor homologs have been identified through the homology search of genome databases (Davis *et al.*, 2002) and two of them, FcRH1 and FcRH3, are expressed on murine B cells (Davis *et al.*, 2004). In addition, a high affinity Fc receptor for IgA and IgM (Fcα/μR) has been identified on B cells as well as on non-lymphoid cells. This receptor acquires the ability to bind IgM upon B cell activation and can mediate endocytosis of IgM-coated bacteria (Shibuya *et al.*, 2000; Sakamoto *et al.*, 2001). The functions of these novel Fc receptors remain to be determined.

IgG-mediated enhancement of T-D responses

Formation of IgG-containing ICs with soluble protein antigens has an enhancing effect on T-D antibody responses (Heyman, 2003). This effect is mediated by IgG_1, IgG_{2a}, and IgG_{2b} and is independent of complement (Wiersma *et al.*, 1990). IgG-mediated enhancement is impaired in FcRγ chain-deficient mice (Wernersson *et al.*, 1999). Although both FcγRI and FcγRIII rely on the FcRγ chain for their surface expression and signal transduction, the enhancement is normal in mice lacking FcγRIII, implying a selective involvement of FcγRI in this process. Interestingly, $Fc\gamma RIIB^{-/-}$ mice show an

over-enhancement of IgG-mediated enhancement (Wernersson *et al.*, 1999), suggesting that the inhibitory signaling via FcγRIIB counterbalances the activating effect of FcγRI signaling in regulation of antibody responses to T-D antigens.

IgG-mediated suppression of T-I type II responses

Passive transfer of immune serum into naïve recipients suppresses antibody responses against T-I type II or haptenated particulate antigens administered in close temporal proximity to serum transfer (Heyman, 2003). This suppressing activity of immune serum is mediated by antigen-specific IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3, but not other isotypes (Brüggemann and Rajewsky, 1982) and is independent of complement engagement (Heyman *et al.*, 1988). The precise mechanism of IgG-mediated suppression of T-I type II responses is unknown. Although isotype selectivity implies the involvement of an Fcγ receptor, known Fcγ receptors do not appear to be involved in this process (Karlsson *et al.*, 1999; Karlsson *et al.*, 2001).

B Cell Memory

Central to the phenomenon of acquired immunity is the concept of immunologic memory—i.e., the ability of the immune system to generate an enhanced antibody response against previously encountered antigens. In his work on staphylococcal toxoid, Burnet reported that the primary antibody response was slow and of low titer, whereas the secondary response rose rapidly to a higher titer (Burnet, 1940). In similar experiments, secondary immunization of guinea pigs with a bacteriophage ϕX 174 resulted in accelerated production of anti-phage antibodies in the serum. Furthermore, secondary serum showed 10-100 fold enhancement in phage-neutralizing capacity compared with the serum obtained after primary immunization with the same dose of the bacteriophage (Uhr *et al.*, 1962).

It has been observed that serum affinity against T-D antigens increases progressively with time after immunization in rabbits and guinea pigs (Eisen and Siskind, 1964; Siskind *et al.*, 1968; Goidl *et al.*, 1968). Such affinity maturation of antibody responses appears to be due to somatic diversification of immunoglobulin V regions, first uncovered by the analysis of the amino acid sequences of Igλ chains from several murine plasmacytoma-derived antibodies (Weigert *et al.*, 1970).

Comparison of the nucleotide sequence of an Igλ chain cloned from a mouse embryo with that from an Igλ-secreting tumor cell line revealed that the latter carried somatic mutations (Bernard *et al.*, 1978). Further detailed analysis of antibody affinities and nucleotide sequences of immunoglobulin V regions from hybridoma clones after primary and secondary immunization with haptenated T-D antigens, such as nitrophenylacetyl (Reth *et al.*, 1978; Cumano and Rajewsky, 1985; Cumano and

Rajewsky, 1986) or phenyloxazolone (Griffiths *et al.*, 1984; Berek *et al.*, 1985), confirmed that somatic hypermutation is the molecular basis for affinity maturation of the antibody response.

The clonal selection theory attributes immunologic memory to the formation of quiescent "memory" cells derived from clonally expanded precursors during the primary response and capable of giving rise to antibody-producing plasma cells upon secondary activation. The existence of the quiescent cellular component of B cell memory was first demonstrated by Okumura *et al.*, who showed that FACS-purified IgG^+ B cells from primed mice develop into IgG-secreting plasma cells upon adoptive transfer and immunization with the same antigen (Okumura *et al.*, 1976). Since IgG^+ B cells constitute less than 1% of all B cells, this experiment raised a question of how such a small cellular compartment is able to contribute to robust IgG humoral responses. Martin and Goodnow resolved this paradox by demonstrating that the membrane tail of IgG enhances the extrafollicular proliferative burst of B cells (Martin and Goodnow, 2002).

T-D memory B cells are characterized by somatic mutations in their immunoglobulin V regions (Siekevitz *et al.*, 1987; Berek *et al.*, 1987), by increased affinity to antigen (Hayakawa *et al.*, 1987), and by the lack of IgD expression in mice (Black *et al.*, 1978). BrdU studies have revealed that T-D memory B cells are long-lived and are derived from proliferating precursors (Schittek and Rajewsky, 1990). Their long-term survival does not require T cells (Vieira and Rajewsky, 1990) or immunizing antigen (Maruyama *et al.*, 2000). Although memory B cells are typically $B220^+$, a distinct compartment of $B220^-$ memory B cells with somatically mutated V regions, elicited by T-D antigens administered in Ribi adjuvant (a TLR4 ligand), has been

described in mice (McHeyzer-Williams *et al.*, 2000) and shown to accumulate both in the spleen and in the bone marrow (Driver *et al.*, 2001).

In humans, memory B cells are characterized by the expression of CD27 (Agematsu *et al.*, 1997; Klein *et al.*, 1998; Tangye *et al.*, 1998; Agematsu *et al.*, 2000). Both IgM^+ and IgM^- memory B cells have been identified. Human IgM^+ memory B cells found in the blood correspond to splenic MZ B cells by surface marker phenotype and gene expression profile (Weller *et al.*, 2004). The development and/or maintenance of this IgM^+ memory B cell compartment is compromised in splenectomized or asplenic patients, who are prone to infections by encapsulated bacteria and who do not respond to T-independent polysaccharide vaccines (Kruetzmann *et al.*, 2003).

T-independent B cell memory has been observed in the peritoneal B-1b cell compartment in mice. B-1b cells from convalescing mice infected with *B. hermsii* conferred better protection of RAG-1-deficient mice against the challenge with this pathogen than B1b cells from naïve mice. This enhanced protection did not require T cells, somatic hypermutation, or isotype switching (Alugupalli *et al.*, 2003).

It remains controversial whether T-I type II antigens elicit memory B cells. IgG-mediated suppression precludes the analysis of secondary T-I type II responses. Furthermore, very low level of somatic hypermutation during the T-I type II response hinders the identification of T-I memory B cells by analyzing their immunoglobulin V regions. To determine whether T-I type II responses generate memory B cells is the aim of this thesis.

Experimental Approach

A direct method of T-I memory B cell detection became possible with the availability of knock-in mouse strains expressing BCRs of known specificities. In our studies, we used previously generated knock-in mice expressing B1-8 heavy chain variants, B1-8^{high} and B1-8^{low} (Shih *et al.*, 2002). The B1-8 heavy chain (consisting of the V_H186.2, DFL16.1, and J_H2 segments) in combination with the Igλ_1 light chain forms NP-specific BCR (Reth *et al.*, 1978). The B1-8^{high} allele was made by Trp to Leu replacement at codon 33 in the CDR1 of the wild-type B1-8 allele resulting in 10-fold increase in BCR affinity to NP, whereas the B1-8^{low} allele contained four amino acid substitutions that collectively decrease BCR affinity to NP by a factor of 4 relative to the wild-type B1-8 allele (Shih *et al.*, 2002).

A large number of B cells of the same (anti-NP) specificity in B1-8^{high} knock-in mice allowed us to identify memory B cells generated during the immune response against NP-Ficoll. We then confirmed the existence of T-I memory B cells in mice with a normal (non-transgenic) B cell repertoire. Finally, we analyzed T-I memory B cells for their phenotypic and functional properties and identified requirements for their development and regulation of secondary activation.

MATERIALS AND METHODS

Animals

C57BL/6 and TCR$\beta^{-/-}\delta^{-/-}$ mice were purchased from The Jackson Laboratories. $AID^{-/-}$ mice were provided by T. Honjo (Kyoto University, Kyoto, Japan), $PKC\beta^{-/-}$ mice by A. Tarakhovsky (Rockefeller University, New York, NY), $Fc\gamma RIIB^{-/-}$ and $FcR\gamma^{-/-}$ mice by J. Ravetch (Rockefeller University, New York, NY). B1-8^{high} and B1-8^{low} IgH knock-in mice were generated previously and crossed to the CD45.1-allotype C57BL/6 background. Blimp-$1^{flox/flox}$ $CD19^{Cre/+}$ mice (Ohinata *et al.*, 2005) and the Blimp-1^{YFP} reporter strain (provided by E. Meffre, Hospital for Special Surgery, New York, NY) were crossed to B1-8^{high} IgH (CD45.1) knock-in mice.

To create the Blimp-1^{Cre} strain, a bacterial artificial chromosome (BAC) clone #452 P8 (Research Genetics) containing the *prdm-1* gene encoding Blimp-1 was modified using a PCR-based method (Misulovin *et al.*, 2001). The coding region in the first exon of *prdm-1* was substituted by the first 100 bp of the Cre sequence preceded by a nuclear localization sequence. The second exon of *prdm-1* was replaced by the remaining Cre sequence followed by a transcriptional STOP cassette. The modified BAC was linearized and microinjected into pronuclei of zygotes derived from B6CBAF1 mice. Blimp-1^{Cre} mice were crossed to the $ROSA26^{STOP\text{-}EGFP}$ reporter strain (Mao *et al.*, 2001) and to B1-8^{high} IgH (CD45.1) knock-in mice.

FACS analysis

To follow the time course of the T-I type II immune response, B1-8^{high} mice were immunized i.p. with 50 μg of NP_{190}-Ficoll (Biosearchtech) and analyzed on day 0, 1, 2, 3, 4 and 7 after immunization. The following antibodies were used for staining: Ki-67-FITC, CD86-FITC, I-A^b-FITC, Syndecan-1-PE, GL-7-FITC, Fas-PE, Igλ-biotin/Streptavidin-PerCP, and B220-APC (BD Biosciences). All stainings were done in FACS buffer (PBS supplemented with 1% fetal bovine serum, FBS). Before Ki-67 staining, cells were fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) for 30 minutes, and Ki-67 staining was performed in Perm/Wash buffer (BD Biosciences). Similar stainings were done with B1-8^{high} Blimp-$1^{flox/flox}$ $CD19^{Cre/+}$ mice on day 0, 2, and 4 after NP-Ficoll immunization.

For cell surface marker analysis of T-I and T-D memory B cells, $CD45.1^+$ B1-8^{high} B cells were adoptively transferred into wild-type mice, which were immunized i.p. with 50 μg of NP_{190}-Ficoll in PBS or with 50 μg of NP_{16}-CGG (Biosearchtech) in Imject Alum (Pierce). FACS analysis was performed on day 15 after NP-Ficoll immunization or on day 25 after NP-CGG immunization. The following antibodies were used for staining: CD21-FITC, CD23-PE, Igλ-biotin/Streptavidin-PerCP, and CD45.1-APC.

To analyze IgG_1^+ and IgG_3^+ B cells from $TCR\beta^{-/-}\delta^{-/-}$, $PKC\beta^{-/-}$, wild-type, and $AID^{-/-}$ mice, splenocytes were depleted with anti-CD43 and anti-IgM MACS beads (Miltenyi) and stained with CD21-FITC, CD23-PE, Igλ-biotin/Streptavidin-PerCP, and B220-APC. FACS acquisition was done on FACSCalibur (BD Biosciences). CELLQuest (BD Biosciences) was used for FACS data analysis.

FACS sort

To test cells for antibody secretion, CD45.1$^+$ B1-8^{high} B cells were adoptively transferred into wild-type mice, which were immunized i.p. with 50 μg of NP_{190}-Ficoll. On day 15 after immunization, splenocytes were depleted with anti-CD43 MACS beads, stained with B220-FITC, Syndecan-1-PE, Igλ-biotin/Streptavidin-PeCy7, and CD45.1-APC. T-I memory B cells were purified by FACS as B220$^+$ Syndecan-1$^-$ Igλ$^+$ CD45.1$^+$ cells. As a negative control, B220$^+$ Syndecan-1$^-$ Igλ$^+$ B cells were purified from naïve B1-8^{high} mice. As a positive control, B220^{low} Syndecan1$^+$ plasma cells were purified from B1-8^{high} mice on day 4 after i.p. immunization with 50 μg of NP_{190}-Ficoll. FACS sort was performed on FACSVantage SE (BD Biosciences). Sorted cells were analyzed by ELISPOT.

ELISPOT

For ELISPOT assay, plates were coated with 5 μg/ml of NP_{16}-BSA (Biosearchtech) and blocked for 2 hours with RPMI-R5 (RPMI Medium 1640 (Gibco) supplemented with 5% heat-treated FBS, 10mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 55 μM β-Mercaptoethanol). Sorted cells (naïve B cells, memory B cells, and plasma cells) were plated (30,000 cells/well) and incubated overnight in RPMI-R5. Cells were washed away with PBS, and plates were incubated for 2 hours with HRP-conjugated anti-mouse Igλ (Southern Biotechnology). ELISPOTs were developed with AEC reagent (Vector) and analyzed by AID ELISPOT reader (Cell Technology).

Analysis of Blimp-1 expression using Blimp-1^{YFP} reporter mice

To detect Blimp-1 expression during T-I type II response, B1-8^{high} Blimp-1^{YFP} mice were analyzed for YFP expression on day 0, 1, 2, 3, 4 and 15 after i.p. immunization with 50 μg of NP_{190}-Ficoll. Cells were stained for Syndecan-1-PE, Igλ-biotin/Streptavidin-PerCP, and B220-APC.

To detect cells derived from Blimp-1-expressing precursors, splenocytes from B1-8^{high} Blimp-1^{Cre} Rosa26$^{STOP-EGFP}$ (CD45.1) mice were depleted with anti-CD43 MACS beads and sorted to exclude GFP$^+$ cells. $20x10^6$ sorted cells were adoptively transferred into wild-type (B6CBAF1) recipients, which were immunized i.p. with 50 μg of NP_{190}-Ficoll or PBS and analyzed for GFP expression on day 5 and 15 after immunization. Splenocytes were stained with Syndecan-1-PE, Igλ-biotin/Streptavidin-PerCP, and CD45.1-APC.

Analysis of Blimp-1 expression by RT-PCR

B1-8^{high} mice were immunized i.p. with 50 μg of NP_{190}-Ficoll. On day 4, splenocytes were depleted with anti-CD90.2 MACS beads (Miltenyi) and stained with B220-FITC and Syndecan-1-PE to sort B220^{low} Syndecan-1$^+$ plasma cells. On day 15, splenocytes were depleted with anti-CD43 MACS beads (Miltenyi) and stained with B220-FITC, Syndecan1-PE, and Igλ-biotin/Streptavidin-PeCy5 to sort Igλ$^+$ B220$^+$ Syndecan-1$^-$ B cells.

Total RNA was obtained from sorted cells using TRIzol reagent (Invitrogen). RNA was annealed with 0.1 μg/ml of random hexamers by a 5-minute incubation at 72^{0}C followed by incubation at 4^{0}C. cDNA was prepared by adding 200 units of SuperScript

II-RTase (Invitrogen) in the First Strand Buffer (Invitrogen) supplemented with 0.1M DTT and 10 mM dNTPs, and incubating the mix at 42^0 C for 1 hour. The following primers were used for PCR amplification of Blimp-1 and β-actin:

Blimp-1 forward primer: GAAGAAACAGAATGGCAAGA;

Blimp-1 reverse primer: AGTTGCCCTTCAGGT;

β-actin forward primer: CCCTGAAGTACCCCATTGAAC;

β-actin reverse primer: GAAGTCTAGAGCAACATAGCACAGC.

PCR was done on serial (1:5) dilutions of cDNA with HotStarTaq (QIAGEN) at 50^0 C annealing for 33 cycles for Blimp-1 amplification and at 55^0 C annealing for 30 cycles for β-actin amplification.

NP coupling to *S. pneumoniae* cells

S. pneumoniae cells (non-lytical Lyt4-4 variant of an acapsular strain R36A, provided by A. Tomasz, The Rockefeller University, New York, NY) were grown in C+Y medium (Horne *et al.*, 1993) until they reached O.D. of 0.7 corresponding to a density of 10^8 cells per ml. Bacterial cells were pelleted by centrifugation and incubated for 5 minutes with 0.5 mg/ml NP-Succinimide ester, NP-OSu (Biosearchtech). The conjugation reaction was quenched with 1.2 mg/ml glycylglycine (Sigma) in PBS, and the bacteria were washed twice with PBS.

BrdU pulse-chase

In BrdU pulse-chase experiments, splenic B cells from B1-8^{high} or B1-8^{low} mice were purified by negative selection with anti-CD43 MACS beads. Approximately $2x10^7$ purified B cells per recipient mouse were injected i.v. When TCR$\beta^{-/-}\delta^{-/-}$ mice were used as recipients, an additional round of negative selection of B1-8^{high} B cells was done with anti-CD90 MACS beads. Mice were immunized i.p. with 50 μg of NP_{190}-Ficoll in PBS or with 10^8 NP-coupled or noncoupled *S. pneumoniae* cells one day after adoptive transfer, and fed 5-bromo-2'-deoxyuridine (BrdU, Sigma) in the drinking water at a concentration of 0.5 mg/ml on days 1-5 after immunization. When indicated, secondary NP-Ficoll immunization was performed in the absence of BrdU administration preceded or not by second round of adoptive transfer of primed splenocytes into naïve wild-type recipients.

BrdU detection

For BrdU detection, cells that had been first stained with antibodies to cell surface markers (GL7-FITC, Fas-PE, Syndecan-1-PE, B220-PerCP, IgG_3-biotin, Igλ-biotin/Streptavidin-PerCP, and CD45.1-APC) were treated with Cytofix/Cytoperm buffer for 30 minutes, followed by a 10-minute incubation in 10% DMSO (in PBS) and a second incubation in Cytofix/Cytoperm buffer. Cells were then incubated in FACS buffer containing 5 mM $MgCl_2$ and 20 units of DNAse I (Sigma) for 1 hour at 37^0 C, and stained with FITC-conjugated anti-BrdU antibody (BD Biosciences) in Perm/Wash buffer.

CFSE labeling

B cells from naïve B1-8^{high} (CD45.1) and NP-Ficoll primed B1-8^{high} (CD45.1/CD45.2) mice were purified by negative selection with anti-CD43 MACS beads. CFSE-labeling was performed by incubating purified B cells (10^7 cells/ml) in PBS containing 5 μg/ml CFDASE dye for 10 minutes at 37^0 C with subsequent PBS washes. B cells from both groups were mixed together and adoptively transferred into naïve C57BL/6 recipients, which were immunized i.p. with 0, 1, 5, 10, and 50 μg of NP_{190}-Ficoll. CFSE dye dilution was analyzed by FACS on day 5 after immunization in combination with Igλ-PE, CD45.2-biotin/Streptavidin-PerCP and CD45.1-APC staining.

ELISA

Mice were immunized i.p. with 50 μg of NP_{190}-Ficoll, TNP_{44}-Ficoll, or NP_{200}-TNP_{44}-Ficoll (Biosearchtech). When indicated, primary immunization with 1μg of NP_{190}-Ficoll or secondary immunization (on day 42) with 50 μg of NP_{190}-Ficoll was performed. Before adoptive transfer of splenocytes from naïve or NP-Ficoll primed wild-type mice into $PKC\beta^{-/-}$ recipients, splenocytes were stained with Syndecan-1-PE antibody and depleted of Syndecan-1^+ cells by anti-PE MACS beads (Miltenyi).

Mice were bled on day 0, 6, 15, 21, 42, 48, and 57. Serum was collected by centrifuging coagulated blood for 15 minutes at 2,000 rpm. NP-specific antibody titers were measured by sandwich ELISA. High-binding plates (Costar) were coated overnight with 5 μg/ml of NP_2-BSA (Biosearchtech), blocked for 1 hour with PBS containing 0.2% Tween-20 and 1% BSA, incubated with serial (1:3) serum dilutions for 2 hours, followed by a 2-hour incubation with HRP-conjugated anti-mouse IgM or IgG (Jackson

ImmunoResearch Laboratories). ELISA plates were developed using 1-Step ABTS reagent (Pierce) and read at the wavelength of 405 nm by VERSAmax microplate reader (Molecular Devices). Titers were calculated using SOFTmax Pro (Molecular Devices) and expressed in arbitrary units relative to an immune serum sample that was included in all plates.

Anti-NP IgG antibody production

The 9T13 hybridoma cell line provided by T. Azuma (Tokyo University of Science, Chiba, Japan) was the source of anti-NP IgG_1 antibody. 9T13 hybridoma was grown for 5-6 days in DMEM Medium (Gibco) supplemented with 10% heat-treated ultra-low IgG FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin. Cells were spun down and supernatants were filtered and incubated overnight with 1 ml/L of protein G Sepharose beads (GE Healthcare). Beads were loaded on the column (1.5 ml of beads per column) and washed with PBS. Antibody was eluted with 4.5 ml of 0.1M Glycine buffer (pH 3) and neutralized with 0.5 ml of 1M Tris buffer (pH 8). Purified antibody was dialyzed overnight against PBS. Isotype control antibody (13C4) was purchased from the Rockefeller Monoclonal Antibody facility. 0.4 mg of each antibody was injected i.v. one day before secondary NP-Ficoll immunization of $AID^{-/-}$ mice.

RESULTS

PART I: PRIMARY T-INDEPENDENT TYPE II IMMUNE RESPONSE

Time-course of T-I type II B cell response

The time course of the T-I type II B cell response was analyzed in B1-8^{high} mice immunized with NP-Ficoll. Splenic NP-specific Igλ^+ B1-8^{high} B cells upregulated a proliferation marker Ki-67 as early as day 1 after immunization. The peak of B cell expansion and expression of activation markers CD86 and I-A^b was observed on day 2. Syndecan-1^+ plasma cells appeared on day 3, and GL-7^+ Fas^+ T-I germinal centers—on day 4 after immunization. By day 7, no proliferating or activated cells could be detected (Figure 2).

T-I type II immune response generates memory B cells

We sought to determine whether NP-Ficoll immunization elicits memory B cells. Assuming that quiescent memory B cells are derived from proliferating antigen-experienced precursors, we used an *in vivo* BrdU pulse-chase strategy for their detection. To ensure that the analysis was not confounded by BrdU incorporation into dividing bone marrow B cell precursors, we adoptively transferred allotype-marked (CD45.1) splenic B cells from B1-8^{high} IgH knock-in mice into naïve wild-type recipients before immunization and subsequently analyzed only the transferred population. Recipient mice were immunized with NP-Ficoll and fed BrdU during the proliferative phase of the T-I type II response (days 1-5), after which BrdU was withdrawn. Incorporation of BrdU

into dividing B cells was assessed by FACS analysis immediately after BrdU withdrawal. To detect quiescent long-term survivors derived from proliferating precursors, BrdU retention was assayed on day 15, 60, and 120 (Figure 3A). We detected allotype-marked BrdU-labeled B cells in the spleen of NP-Ficoll immunized recipients, but not in control recipients injected with PBS and fed BrdU (Figure 3B). BrdU-labeled B cells were $Ig\lambda^+$ (Figure 3B) and thus NP-specific (Reth *et al.*, 1978; Shih *et al.*, 2002). Because subsequent cell division in the absence of BrdU (days 5-120) would have resulted in loss of BrdU by dilution, the detected BrdU-labeled cells must be quiescent.

The above BrdU pulse-chase strategy might have labeled long-lived terminally differentiated plasma cells and/or quiescent memory B cells. To distinguish between these cell types, we analyzed BrdU-labeled cells for the expression of a plasma cell marker (Syndecan-1) and for antibody secretion. The majority of BrdU-labeled cells expressed a B cell marker (B220), although at lower levels than naïve B cells, and were Syndecan-1^- (Figure 3C). To make sure that BrdU-labeled cells did not secrete antibodies, we purified $Ig\lambda^+$ B1-8^{high} B cells by FACS on day 15 after adoptive transfer and NP-Ficoll immunization and tested them for antibody secretion by ELISPOT. They did not secrete antibodies (Figure 3D). Consistent with the possibility that BrdU-labeled cells were memory B cells, 8% expressed a secondary Ig isotype (IgG_3) on their surface (Figure 3C). We conclude that quiescent BrdU-labeled $B220^{low}$ Syndecan-1^- $Ig\lambda^+$ B1-8^{high} B cells formed during the immune response to NP-Ficoll are memory B cells.

NP-Ficoll elicits memory B cells in the absence of T cells

Immune responses against polysaccharides do not require T cells for antibody production (Mond *et al.*, 1995). To test T cell requirement for the development of memory B cells against polysaccharides, we repeated BrdU pulse-chase in T cell-deficient mice. Allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into TCR$\beta^{-/-}\delta^{-/-}$ recipients, which were immunized with NP-Ficoll or PBS and fed BrdU. To ensure that the transferred B cell sample was not contaminated with T cells, we performed double depletion of T cells by CD90-MACS beads and confirmed the purity of the sample by CD3 staining (not shown). BrdU-labeled Igλ^+B1-8^{high} memory B cells were detected in TCR$\beta^{-/-}\delta^{-/-}$ recipients on day 15 after NP-Ficoll immunization (Figure 4). Thus, T cells are not required for the development of memory B cells elicited by NP-Ficoll.

NP-specific memory B cells are elicited by NP-coupled *S. pneumoniae*

Pathogens that induce T-I type II responses include bacteria such as *S. pneumoniae*. We tested whether, similar to NP-Ficoll, antigens carried on the bacterial cell surface elicit memory B cells. Allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were immunized with NP-coupled or non-coupled *S. pneumoniae* and fed BrdU. The immune response against NP-*S. pneumoniae,* but not against non-coupled bacteria, generated NP-specific (Igλ^+) memory B cells detected by BrdU staining on day 15 after immunization (Figure 5). Therefore, pathogens that induce T-I type II immune responses stimulate production of memory B cells.

T-I memory B cells resemble naïve B cells in their lifespan and sensitivity to polysaccharide antigens

To estimate the lifespan of T-I memory B cells, we compared their survival to that of naïve B cells. Adoptively transferred naïve Igλ^+B1-8^{high} B cells initially constituted 0.27% of total splenocytes, and in the absence of immunization their number declined gradually, reaching half of the initial input in approximately 90 days (Figure 6). NP-Ficoll immunization resulted in 10-fold expansion of this population by day 5, followed by a sharp decline from 2.7% to 0.7% of total splenocytes between day 5 and 30 (Figure 6). This loss of Igλ^+B1-8^{high} B cells in immunized recipients was disproportionate to the loss of Igλ^+B1-8^{high} B cells in naïve recipients during the same time period (from 0.27% to 0.22%). After day 30, however, the half-life of the Igλ^+B1-8^{high} B cell population was equivalent in immunized and naïve recipients. Notably, Igλ^+B1-8^{high} B cells remained more numerous in immunized than in naïve recipients for up to 4 months. These results suggest that after the initial expansion and loss of B cells responding to T-I type II antigens, an expanded long-lived population of memory B cells is maintained with a half-life similar to that of naïve B cells (approximately 90 days).

We next compared the sensitivity of naïve and T-I memory B cells to various doses of NP-Ficoll. B cells from naïve or NP-Ficoll primed B1-8^{high} IgH knock-in mice expressing CD45.1 or CD45.1/CD45.2 allotypes, respectively, were purified, labeled with CFSE, and adoptively transferred into wild-type recipients. NP-Ficoll priming was done 30 days before the adoptive transfer. The proliferative response of transferred B1-8^{high} B cells was assessed by CFSE dilution on day 5 after NP-Ficoll immunization of recipients (Figure 7A). We found no difference between naïve and primed Igλ^+ B1-8^{high}

B cells in their responsiveness to doses of NP-Ficoll ranging from 1 to 50 μg (Figure 7B). This suggests that naïve and T-I memory B cells of the same antigen affinity have similar sensitivity to polysaccharide antigens.

T-I and T-D memory B cells have distinct cell surface marker phenotypes

To determine whether there are phenotypic differences between T-I and T-D memory B cells, we examined their cell surface marker phenotype. B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were immunized either with NP-Ficoll or with a T-D antigen NP-chicken γ globulin (NP-CGG) (Figure 8A). Igλ^{+} B1-8^{high} memory B cells that developed during T-I or T-D responses differed in the pattern of CD21 versus CD23 expression: the former expressed low levels of CD21 and CD23, whereas the latter resembled follicular (CD21^{low} CD23^{high}) and MZ (CD21^{high} CD23^{low}) B cells (Figure 8B).

To determine whether such phenotypic differences are found in naturally generated memory B cell populations of T-I or T-D origin, we analyzed the phenotype of memory B cells identified by surface expression of IgG_1+IgG_3 in aged TCRβ$^{-/-}$δ$^{-/-}$, PKCβ$^{-/-}$, and wild-type mice (Figure 9). IgG^{+} memory B cells in TCRβ$^{-/-}$δ$^{-/-}$ mice, which can generate only T-I responses, showed the pattern of CD21 versus CD23 expression similar to that of Igλ^{+} B1-8^{high} memory B cells derived from NP-Ficoll immunization. In contrast, PKCβ$^{-/-}$ mice, which can generate only T-D responses (Leitges *et al.*, 1996), accumulated IgG^{+} memory B cells resembling Igλ^{+} B1-8^{high} memory B cells derived from NP-CGG immunization. Finally, in wild-type mice IgG^{+} B cells with the T-I memory phenotype (CD21^{low} CD23^{low}) constituted about 50% of the total IgG^{+} B cell pool. These results indicate that naturally arising T-I memory B cells

show the same surface phenotype as $Ig\lambda^+$ B1-8^{high} memory B cells that develop in response to NP-Ficoll immunization. Furthermore, T-I memory B cells are phenotypically distinct from conventional T-D memory B cells and constitute a significant portion of the total IgG^+ memory B cell pool.

BCR affinity regulates development of T-I memory B cells

To determine the effect of BCR affinity on the development of T-I memory B cells, we performed BrdU pulse-chase using B1-8^{low} B cells, whose BCR affinity to NP is 4-fold lower than that of B cells expressing the wild-type B1-8 IgH allele. Allotype-marked (CD45.1) B1-8^{low} B cells were adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll or PBS and fed BrdU. BrdU incorporation into B1-8^{low} B cells was assayed on day 5 after NP-Ficoll immunization, and BrdU retention by B1-8^{low} memory B cells—on day 15 (Figure 10A). B1-8^{low} B cells responded to NP-Ficoll immunization but failed to develop the memory compartment (Figures 10B and 10C).

B1-8^{low} B cells might have been unable to develop the memory compartment either due to the intrinsically low affinity of their antigen receptor or due to the competition with B cells of higher affinity to NP present in wild-type recipients. To distinguish between these possibilities, we next used $PKC\beta^{-/-}$ mice as recipients of B1-8^{low} B cells. Because of the B cell-autonomous defect in T-I type II antibody responses in $PKC\beta^{-/-}$ mice (Leitges *et al.*, 1996), the development of the B1-8^{low} memory B cell compartment in response to NP-Ficoll immunization would occur in the absence of potential competition with endogenous B cells. BrdU-labeled B1-8^{low} memory B cells were detected in $PKC\beta^{-/-}$ recipients on day 15 after NP-Ficoll immunization (Figure 10B).

We conclude that affinity-based competition, rather than intrinsic antigen affinity of the BCR, regulates the development of the T-I memory B cell compartment.

Blimp-1 expression is restricted to plasma cells during T-I type II immune response

Attempting to identify factors involved in T-I memory B cell development, we tested the formation of T-I memory B cells by means of BrdU pulse-chase in a number of knock-out strains crossed to B1-8^{high} IgH knock-in mice. We found that B cell deficiency in a transcription factor Blimp-1 affected the formation or detection of BrdU-labeled memory B cells in response to NP-Ficoll immunization (data not shown). We therefore examined the role of Blimp-1 in the T-I type II B cell response.

First, we analyzed Blimp-1 expression during the course of the T-I type II response in Blimp-1^{YFP} reporter mice crossed to the B1-8^{high} IgH knock-in strain. Mice were immunized with NP-Ficoll, and YFP expression, representing Blimp-1 expression, was analyzed by FACS on day 0, 1, 2, 3, 4, and 15 after immunization. YFP$^+$ Syndecan-1$^+$ cells were detected on day 3 and 4 after immunization. No other YFP$^+$ population could be detected throughout the course of the T-I type II B cell response (Figure 11A). Blimp-1 expression by purified Syndecan-1$^+$ plasma cells on day 4, but not by NP-specific B cells on day 15, was confirmed by RT-PCR (Figure 11B).

Blimp-1-deficient B cells respond to NP-Ficoll immunization but fail to give rise to plasma cells

To examine the effect of Blimp-1 deficiency on T-I type II responses, B1-8^{high} or B1-8^{high} Blimp-1$^{flox/flox}$ CD19$^{Cre/+}$ mice were immunized with NP-Ficoll and their splenocytes were

analyzed on day 0, 2, and 4 after immunization for proliferation, expression of activation markers, and the formation of plasma cells and germinal centers. B1-8^{high} Blimp-$1^{flox/flox}$ $CD19^{Cre/+}$ mice showed normal proliferation and activation of NP-specific ($Ig\lambda^+$) B cells on day 2 after NP-Ficoll immunization, but had a significantly reduced number of Syndecan-1^+ plasma cells and an increased number of GC B cells on day 4 (Figure 12). Thus, Blimp-1 deficiency compromises plasma cell development, but does not appear to affect B cell activation in response to NP-Ficoll immunization.

T-I memory B cells are not derived from Blimp-1-expressing precursors

Next, we performed genetic tracing using the Cre-lox recombination strategy to test whether T-I memory B cells are derived from Blimp-1 expressing precursors. Cre-recombinase was introduced into a BAC containing the *prdm-1* gene encoding Blimp-1. Blimp-1^{Cre} BAC-transgenic mice were bred with the $Rosa26^{STOP\text{-}EGFP}$ reporter strain with a floxed transcriptional STOP cassette (Mao *et al.*, 2001). In this system, Cre-recombinase expression leads to the excision of the STOP cassette in Blimp-1-expressing cells. Subsequent expression of GFP in all progeny cells is independent of further Blimp-1 expression (Figure 13).

A fraction of B cells in Blimp-1^{Cre} $Rosa26^{STOP\text{-}EGFP}$ mice were GFP positive in the absence of any immunization (not shown). GFP^- B cells were purified by FACS from B1-8^{high} Blimp-1^{Cre} $Rosa26^{STOP\text{-}EGFP}$ mice expressing CD45.1 allotype and adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll or PBS. GFP expression was analyzed on day 5 and 15 after immunization (Figure 14A). At both time points, immunization-specific GFP expression was restricted to Syndecan-1^+ plasma

cells (Figure 14B). The majority of Igλ^+ B1-8^{high} memory B cells, detected on day 15 after NP-Ficoll immunization, did not express GFP and thus were not derived from Blimp-1-expressing precursors.

Figure 2. Immune response to NP-Ficoll.

Ki-67, CD86, I-A^b, B220 versus Syndecan-1, and Fas versus GL-7 staining of splenocytes from NP-Ficoll immunized B1-8^{high} mice analyzed at the indicated time points. Broken lines in histogram overlays represent levels of Ki-67, CD86 and I-A^b expression in naïve mice. Gated on Igλ^+ (NP-specific) B cells.

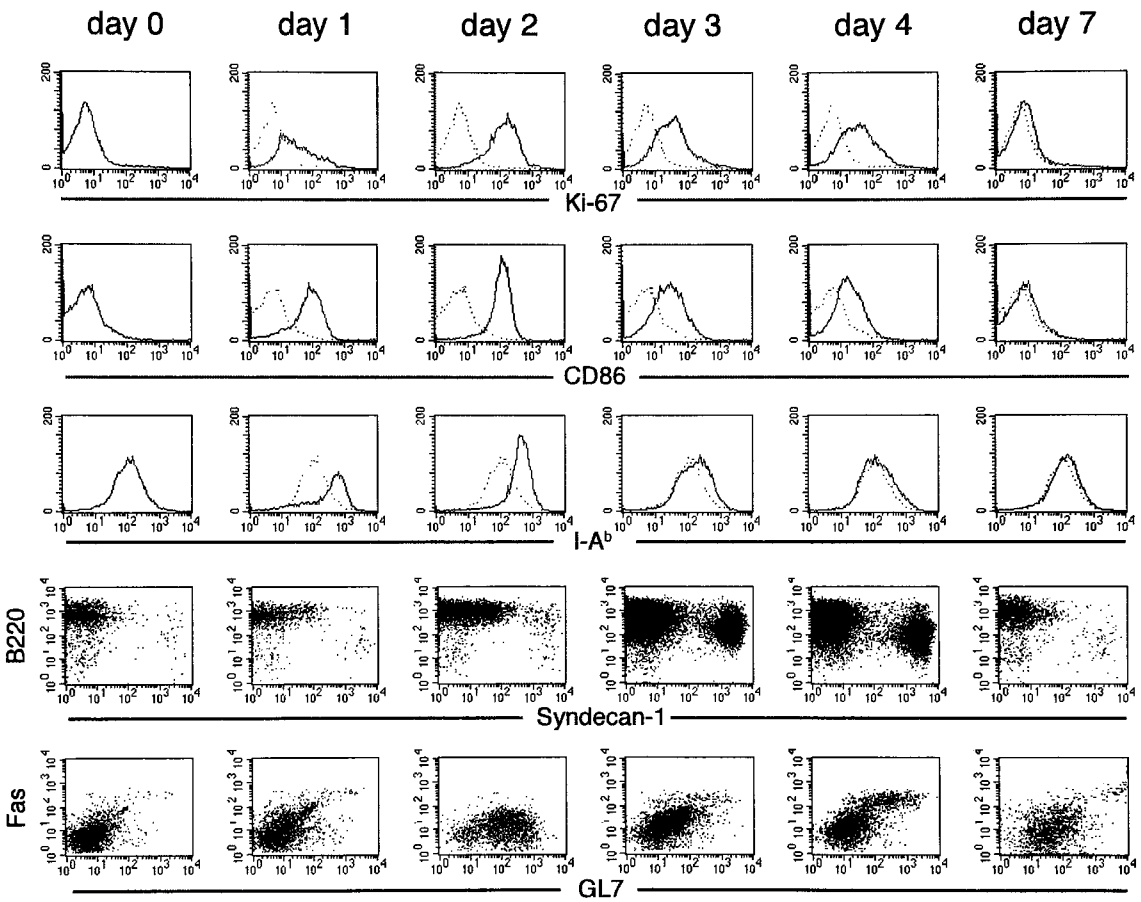

day 0
day 1
day 2
day 3
day 4
day 7
Ki-67
CD86
I-Ab
B220
Syndecan-1
Fas
GL7

Figure 3. NP-Ficoll immunization elicits memory B cells.

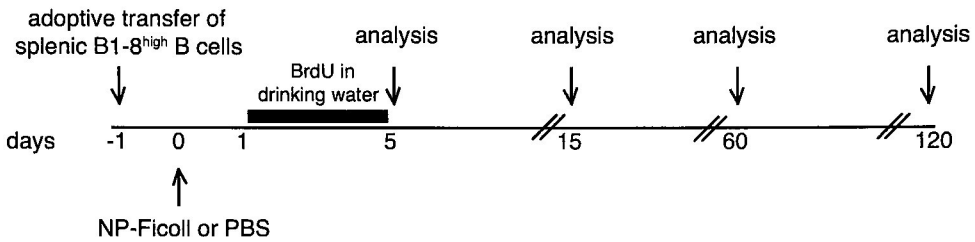
(A). BrdU pulse-chase strategy. Allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll or PBS and fed BrdU in drinking water (days 1-5). Incorporation/retention of BrdU was assayed on day 5, 15, 60, and 120.

(B). BrdU staining of adoptively transferred B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from NP-Ficoll immunized or naïve wild-type recipients analyzed at the indicated time points. Gated on CD45.1^+ cells.

(C). B220 versus Syndecan-1 and B220 versus IgG_3 staining of B1-8^{high} B cells from naïve recipients on day 15 after adoptive transfer (left) and of BrdU-gated B1-8^{high} B cells from NP-Ficoll immunized recipients on day 15 after adoptive transfer and immunization (right). Gated on CD45.1^+ cells.

(D). ELISPOT assay for antibody secretion by purified naïve B cells, memory B cells, and plasma cells. Naïve B cells (Igλ$^+$ B220$^+$ Syndecan-1^-) were purified by FACS from B1-8^{high} mice; memory B cells (Igλ$^+$ B220^{low} Syndecan-1^- CD45.1^+) were purified from wild-type mice, 15 days after they received allotype-marked (CD45.1) B1-8^{high} B cells and were immunized with NP-Ficoll; plasma cells (Igλ$^+$ B220^{low} Syndecan-1^+) were purified from B1-8^{high} mice on day 4 after NP-Ficoll immunization.

A.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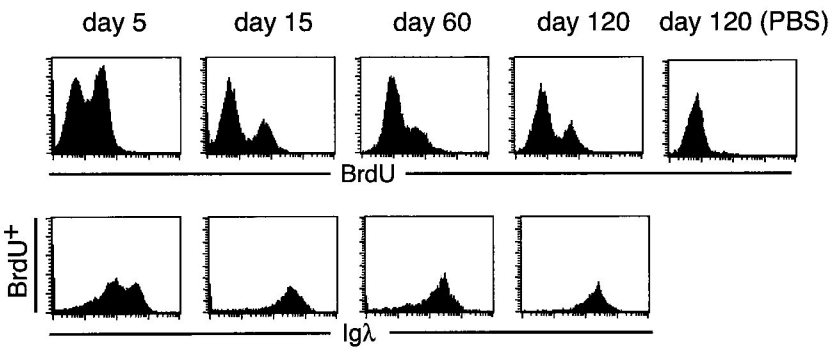
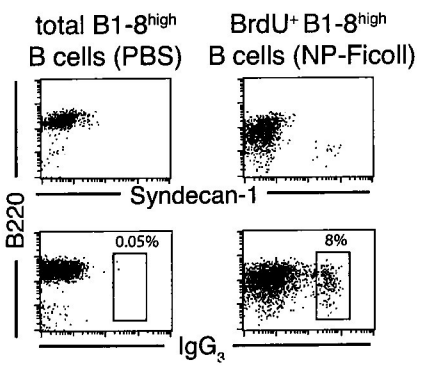
analysis
analysis
analysis
BrdU in
drinking water
days
-1
0
1
5
15
60
120
NP-Ficoll or PBS

B.


day 5
day 15
day 60
day 120
day 120 (PBS)
BrdU
BrdU+
Igλ

C.


total B1-8high
B cells (PBS)
BrdU+ B1-8high
B cells (NP-Ficoll)
Syndecan-1
B220
0.05%
8%
IgGa

D.

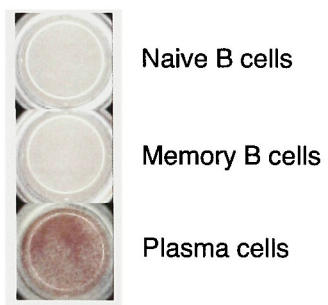

Naive B cells
Memory B cells
Plasma cells

Figure 4. NP-Ficoll can elicit memory B cells in the absence of T cells.

BrdU pulse-chase was performed as indicated in Figure 2A, except that $TCR\beta^{-/-}\delta^{-/-}$ mice were used as recipients, and the experiment was carried out until day 15. Histograms represent BrdU staining of adoptively transferred B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from NP-Ficoll immunized or naïve $TCR\beta^{-/-}\delta^{-/-}$ recipients analyzed at the indicated time points. Gated on $CD45.1^{+}$ cells.

B1-8^{high} B cells ---> TCR$\beta^{-/-}\delta^{-/-}$ mice

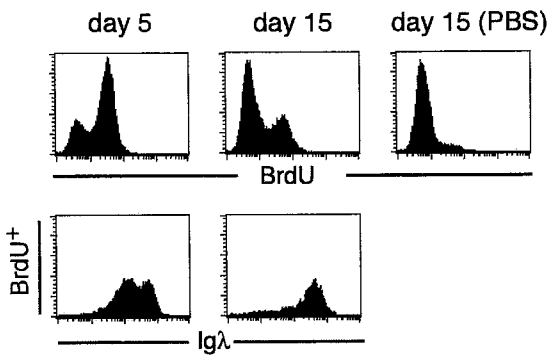


Figure 5. NP-conjugated *S. pneumoniae* elicit NP-specific memory B cells.

BrdU pulse-chase was performed as indicated in Figure 2A, except that recipient mice were injected with NP-conjugated or unconjugated *S. pneumoniae* cells, and the experiment was carried out until day 15. Histograms represent BrdU staining of adoptively transferred B1-8^{high} B cells (top) and Igλ staining of BrdU-gated B1-8^{high} B cells (bottom) from wild-type recipients immunized with NP-coupled (NP-Strep) or non-coupled (Strep) *S. pneumoniae* and analyzed at the indicated time points. Gated on CD45.1^{+} cells.

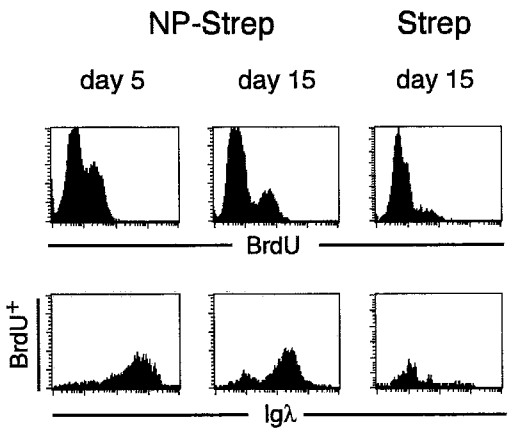

NP-Strep
Strep
day 5
day 15
day 15
BrdU
BrdU+
Igλ

Figure 6. Survival of adoptively transferred Igλ^+ B1-8^{high} B cells in NP-Ficoll immunized and naïve wild-type recipients.

Wild-type mice were adoptively transferred with allotype-marked (CD45.1) B1-8^{high} B cells and immunized with NP-Ficoll or PBS. The number of Igλ^+ B1-8^{high} B cells per total splenocytes is plotted against the time after adoptive transfer. Average values for each time point are plotted as bars.

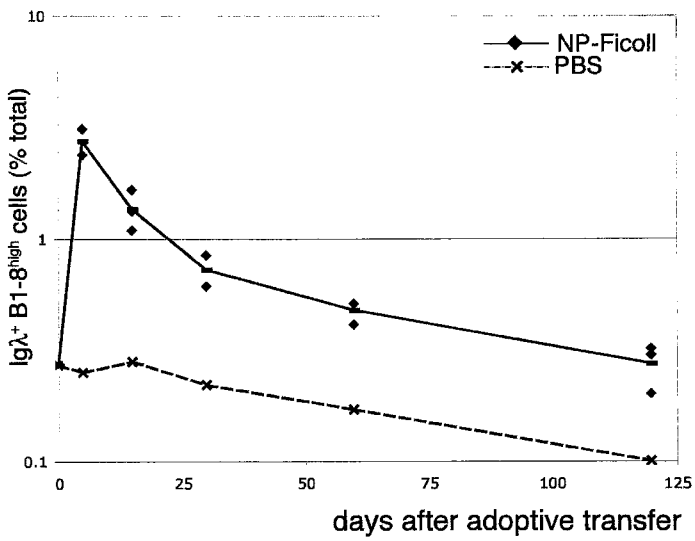
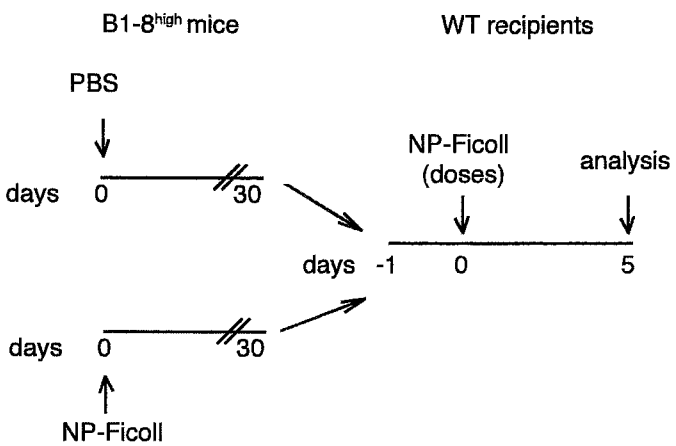

10
1
0.1
NP-Ficoll
PBS
Igλ+ B1-8high cells (% total)
0
25
50
75
100
125
days after adoptive transfer

Figure 7. Similar sensitivity of primed and naïve Igλ$^+$ B1-8^{high} B cells to NP-Ficoll.

(A). Experimental strategy. CD45.1 or CD45.1/CD45.2 B1-8^{high} mice were primed with PBS or NP-Ficoll, respectively. On day 30 after priming, B cells from both groups were purified, labeled with CFSE, and co-transferred into naïve wild-type (CD45.2) recipients, which were immunized with a range of NP-Ficoll doses.

(B). CFSE dilution by adoptively transferred naïve or NP-Ficoll primed Igλ$^+$ B1-8^{high} B cells on day 5 after NP-Ficoll immunization. Doses of NP-Ficoll used for immunization are indicated. Gated on CD45.1$^+$ (naïve) or CD45.1$^+$ CD45.2$^+$ (primed) Igλ$^+$ (NP-specific) B cells.

A.


B1-8high mice
WT recipients
PBS
days 0 30
NP-Ficoll (doses)
analysis
days -1 0 5
days 0 30
NP-Ficoll

B.

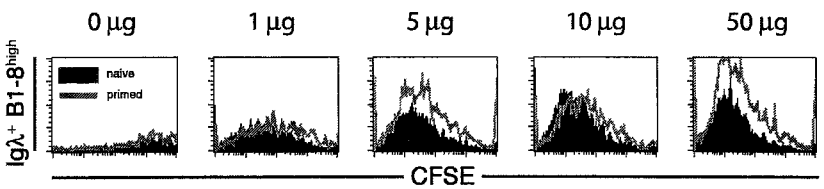
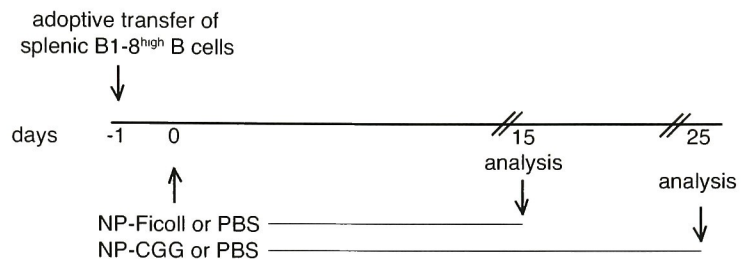

0 µg
1 µg
5 µg
10 µg
50 µg
naive
primed
Igλ+ B1-8high
CFSE

Figure 8. Distinct phenotypes of memory B cells elicited by NP-Ficoll versus NP-CGG.

(A). Experimental strategy. Allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll or NP-CGG. FACS analysis was done on day 15 after NP-Ficoll immunization and on day 25 after NP-CGG immunization.

(B). CD21 versus CD23 staining of adoptively transferred Igλ^+ B1-8^{high} B cells from mice immunized with NP-Ficoll or NP-CGG. Gated on CD45.1$^+$ Igλ^+ (NP-specific) B cells.

A.


adoptive transfer of
splenic B1-8high B cells
days
-1
0
15
analysis
25
analysis
NP-Ficoll or PBS
NP-CGG or PBS

B.

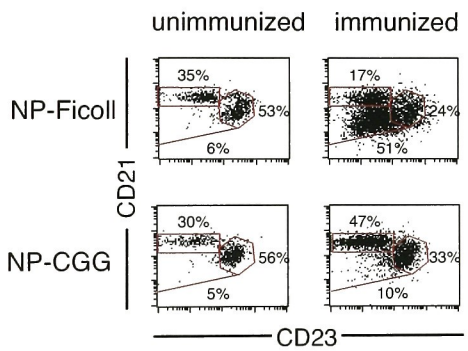

unimmunized
immunized
NP-Ficoll
NP-CGG
CD21
CD23
35%
53%
6%
17%
24%
51%
30%
56%
5%
47%
33%
10%

Figure 9. Phenotype of IgG^+ memory B cells in mice with normal B cell repertoire.

B220 versus IgG_1+IgG_3 staining of splenocytes from $TCR\beta^{-/-}\delta^{-/-}$, $PKC\beta^{-/-}$, wild-type, and $AID^{-/-}$ mice (top) and CD21 versus CD23 staining of IgG_1+IgG_3-gated B cells (bottom). Splenocytes from $AID^{-/-}$ mice deficient in class switch recombination are included as a negative control for anti-IgG staining.

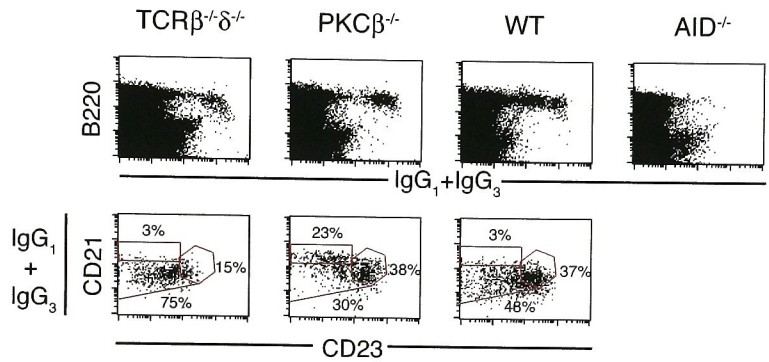

TCRβ-/-δ-/-
PKCβ-/-
WT
AID-/-
B220
IgG1+IgG3
IgG1 + IgG3
CD21
3%
15%
75%
23%
38%
30%
3%
37%
48%
CD23

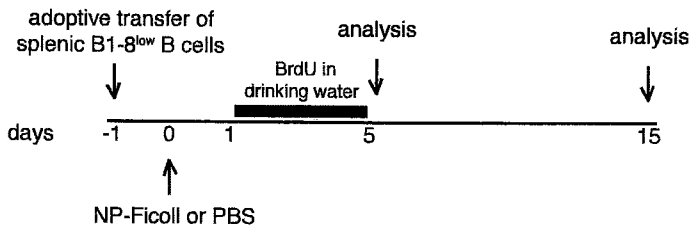
Figure 10. BCR affinity regulates development of T-I memory B cells.

(A). BrdU pulse-chase strategy. Allotype-marked (CD45.1) B1-8^{low} B cells were adoptively transferred into wild-type or PKCβ$^{-/-}$ recipients, which were immunized with NP-Ficoll or PBS and fed BrdU in drinking water (days 1-5). Incorporation/retention of BrdU was assayed on day 5 and 15.

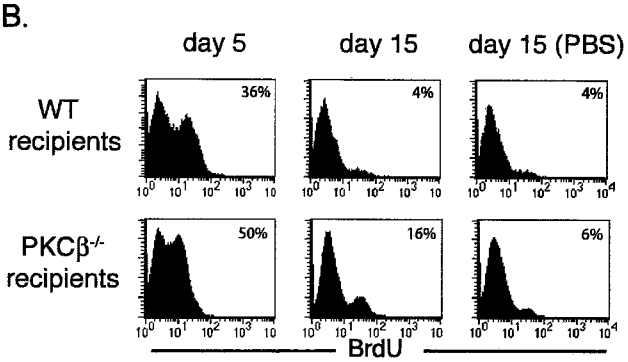
(B). BrdU staining of adoptively transferred B1-8^{low} B cells from wild-type or PKCβ$^{-/-}$ recipients immunized with NP-Ficoll or PBS and analyzed at the indicated time points. Percentages of BrdU^{+} cells are indicated. Gated on CD45.1^{+} cells.

(C). B220 versus Syndecan-1 (left) and Fas versus GL-7 (right) staining of adoptively transferred BrdU-labeled B1-8^{low} B cells on day 5 after NP-Ficoll immunization of wild-type or PKCβ$^{-/-}$ recipients. Gated on CD45.1^{+} cells.

A.


adoptive transfer of
splenic B1-8low B cells
analysis
analysis
BrdU in
drinking water
days
-1
0
1
5
15
NP-Ficoll or PBS

B.


day 5
day 15
day 15 (PBS)
WT
recipients
PKCβ-/-
recipients
36%
4%
4%
50%
16%
6%
BrdU

C.

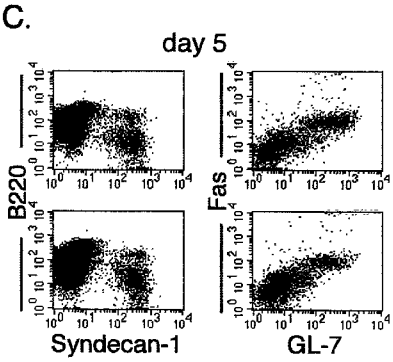
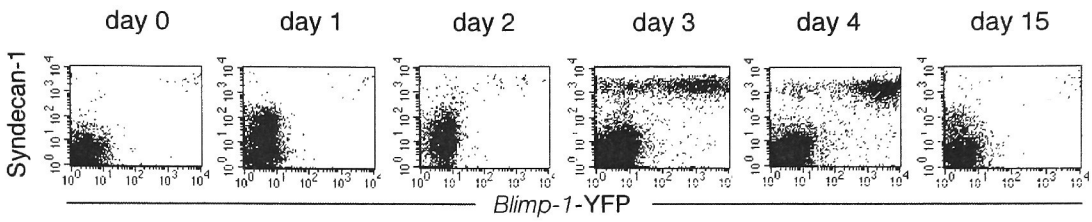

day 5
B220
Fas
Syndecan-1
GL-7

Figure 11. Blimp-1 expression during T-I type II immune response.

(A). FACS analysis of Blimp-1 expression. Syndecan-1 versus *Blimp-1*-YFP staining of splenocytes from NP-Ficoll immunized B1-8^{high} Blimp-1^{YFP} mice analyzed at the indicated time points. Gated on Igλ^+ (NP-specific) B cells.

(B). RT-PCR analysis of Blimp-1 expression. Plasma cells were sorted as B220^{low} Syndecan-1$^+$ cells from splenocytes of B1-8^{high} mice on day 4 after NP-Ficoll immunization. NP-specific B cells were sorted as Igλ^+ B220$^+$ Syndecan-1$^-$ cells from splenocytes of B1-8^{high} mice on day 15 after NP-Ficoll immunization. Blimp-1 and β-actin expression was detected by RT-PCR.

A.


day 0
day 1
day 2
day 3
day 4
day 15
Syndecan-1
Blimp-1-YFP

B.

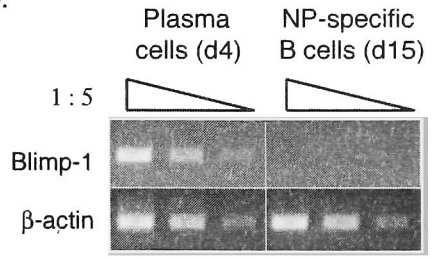

Plasma cells (d4)
NP-specific B cells (d15)
1 : 5
Blimp-1
β-actin

Figure 12. Blimp-1-deficient B cells respond to NP-Ficoll immunization but do not give rise to plasma cells.

Ki-67, CD86, I-A^b, B220 versus Syndecan-1, and Fas versus GL-7 staining of splenocytes from NP-Ficoll immunized B1-8^{high} or B1-8^{high} Blimp-$1^{flox/flox}$ $CD19^{Cre/+}$ mice at the indicated time points. Broken lines in histogram overlays represent levels of Ki-67, CD86 and I-A^b expression in naïve mice. Percentages of plasma cells and GC cells are indicated. Gated on $Ig\lambda^+$ (NP-specific) B cells.

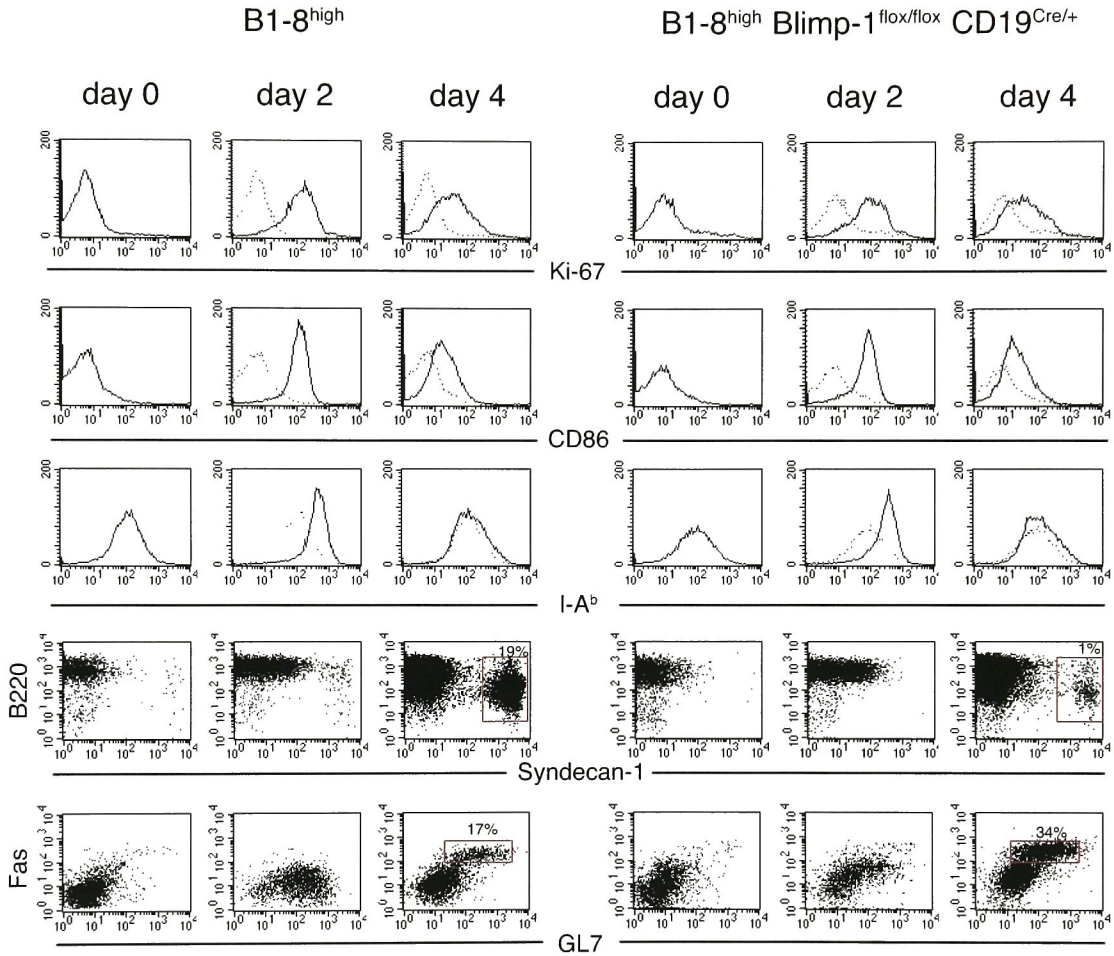

B1-8high
B1-8high Blimp-1flox/flox CD19Cre/+
day 0
day 2
day 4
day 0
day 2
day 4
Ki-67
CD86
I-Ab
B220
19%
1%
Syndecan-1
Fas
17%
34%
GL7

Figure 13. Strategy for genetic tracing of cells derived from Blimp-1-expressing precursors.

Blimp-1^{Cre} BAC-transgenic mice were crossed to the $Rosa26^{STOP\text{-}EGFP}$ reporter strain with a floxed transcriptional STOP cassette. Blimp-1 upregulation results in Cre-recombinase expression, allowing excision of the STOP cassette and leading to constitutive GFP expression under the *Rosa26* promoter independent of further expression of Blimp-1.

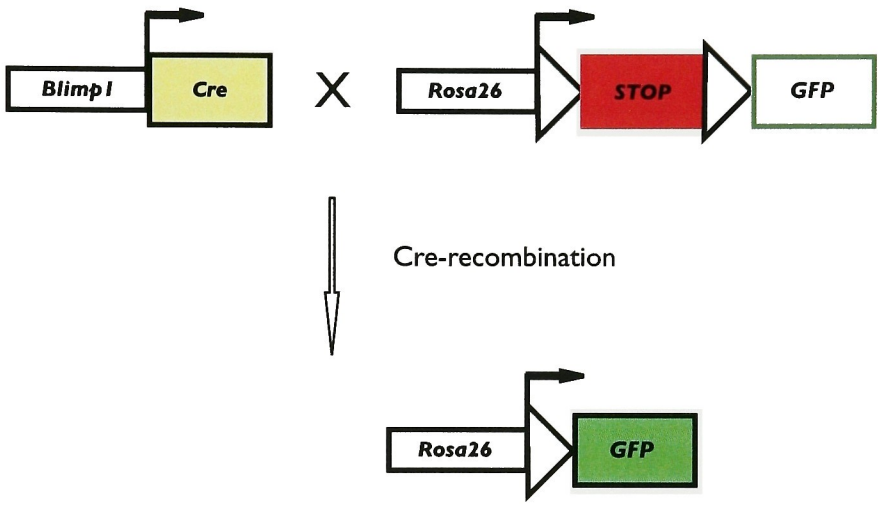
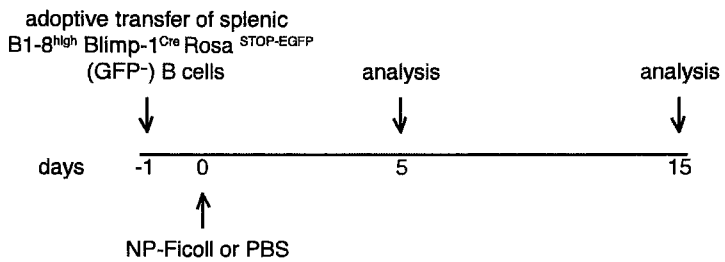

Blimp1
Cre
X
Rosa26
STOP
GFP
Cre-recombination
Rosa26
GFP

Figure 14. T-I memory B cells are not derived from Blimp-1-expressing precursors.

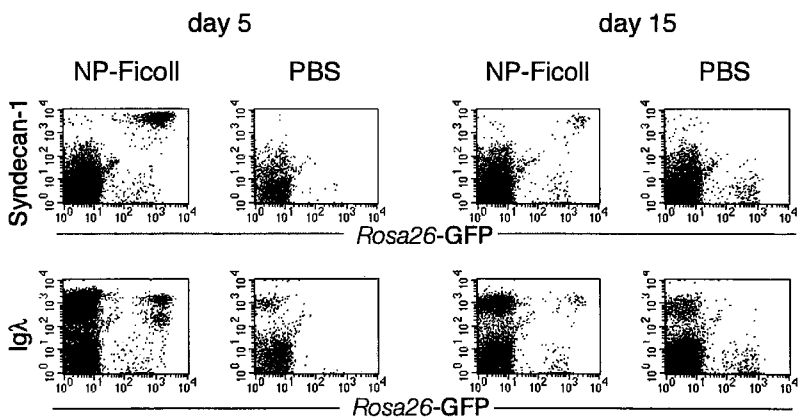
(A). Experimental strategy. Allotype-marked (CD45.1) GFP^- B cells from B1-8^{high} Blimp-1^{Cre} Rosa$26^{STOP\text{-}EGFP}$ mice were purified by FACS and adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll or PBS. GFP expression was analyzed on day 5 and 15.

(B). Syndecan-1 versus *Rosa26*-GFP and Igλ versus *Rosa26*-GFP staining of adoptively transferred B cells analyzed at the indicated time points. Gated on $CD45.1^+$ cells.

A.


adoptive transfer of splenic
B1-8high Blimp-1Cre Rosa STOP-EGFP
(GFP-) B cells
analysis
analysis
days
-1
0
5
15
NP-Ficoll or PBS

B.


day 5
day 15
NP-Ficoll
PBS
NP-Ficoll
PBS
Syndecan-1
Rosa26-GFP
Igλ
Rosa26-GFP

PART II: SECONDARY T-INDEPENDENT TYPE II IMMUNE RESPONSE

Absence of T-I type II recall response in mice primed with high antigen dose

Primary immunization with NP-Ficoll results in a rapid and long-lasting antibody production (Vinuesa *et al.*, 1999). We immunized wild-type mice with 50 or 1 μg of NP-Ficoll, followed by a 50 μg dose 6 weeks later. Mice primed with the low dose of NP-Ficoll responded to secondary immunization with the high dose. However, mice primed with the high dose generated no further increase in anti-NP IgM and IgG titers after secondary immunization compared with those established after primary immunization (Figures 15A and 15B).

Immune serum plays a role in the suppression of T-I type II recall responses (Hosokawa, 1979; Brodeur and Wortis, 1980). We purified splenocytes from naïve or NP-Ficoll primed wild-type mice two weeks after priming, depleted them of Syndecan-1^{+} plasma cells, and adoptively transferred into naïve PKCβ$^{-/-}$ recipients, which have a B cell-autonomous defect in T-I type II responses (Leitges *et al.*, 1996). Recipient mice were immunized with NP-Ficoll or PBS. The recipients of primed splenocytes produced IgM and IgG antibody responses against NP-Ficoll, as did the recipients of naïve splenocytes, whereas control PKCβ$^{-/-}$ mice receiving no cell transfer failed to respond (Figures 16A and 16B). These results confirm that B cells are unresponsive to secondary immunization with NP-Ficoll in primed mice, but their responsiveness is restored upon adoptive transfer into naïve mice.

Antigen-specific IgG antibodies regulate activation of T-I memory B cells

We next tested whether T-I memory B cells respond to polysaccharide antigens. To generate and label T-I memory B cells, allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into wild-type mice, which were immunized with NP-Ficoll and fed BrdU (days 1-5). On day 20, splenocytes were adoptively transferred into a second group of naïve wild-type recipients, which were injected with PBS or immunized with NP-Ficoll (Figure 17A). Alternatively, NP-Ficoll primed mice were injected with PBS or re-immunized with NP-Ficoll on day 20 after primary immunization (Figure 17C). By day 5 after secondary immunization with NP-Ficoll, but not with PBS, BrdU-labeled memory B cells that had been adoptively transferred into naïve recipients lost BrdU due to their proliferation leading to BrdU dilution (Figure 17B). In contrast, BrdU-labeled memory B cells that had not been adoptively transferred into naïve recipients retained BrdU (Figure 17D), thus not responding to secondary immunization with NP-Ficoll. Remarkably, this inhibition of memory B cell activation in response to secondary NP-Ficoll immunization was not observed in AID$^{-/-}$ mice (Figure 17D), deficient in class switch recombination (Muramatsu *et al.*, 2000). These results indicate that T-I memory B cells are responsive to secondary immunization with polysaccharide antigens; however, their activation is suppressed in primed wild-type mice but not in AID-deficient or naïve mice.

To determine whether antigen-specific IgG antibodies account for the suppression of the T-I type II recall response, we first tested the recall response to NP-Ficoll in AID$^{-/-}$ mice, which lack IgG (Muramatsu *et al.*, 2000). Secondary immunization boosted NP-specific IgM titers in AID-deficient but not in wild-type mice (Figure 18A). Next, we

injected $AID^{-/-}$ mice with NP-specific IgG, non-specific IgG, or PBS one day before secondary NP-Ficoll immunization. Only NP-specific antibody suppressed the recall response to NP-Ficoll in $AID^{-/-}$ mice (Figure 18B). We conclude that antigen-specific IgG antibodies are necessary and sufficient for the suppression of T-I type II recall responses.

IgG-mediated suppression of T-I type II immune response does not depend on epitope masking

To test whether epitope masking accounts for the suppression of T-I type II immune responses by antigen-specific antibodies, wild-type mice were primed with TNP-Ficoll or PBS and two weeks later immunized with NP-Ficoll or doubly-conjugated NP/TNP-Ficoll. As expected, TNP-Ficoll priming did not affect the antibody response against NP-Ficoll. In contrast, TNP-Ficoll priming caused a 6-fold reduction in anti-NP antibody titers in response to NP/TNP-Ficoll immunization relative to the PBS priming control (Figures 19A and 19B). These results indicate that subsequent T-I type II immune responses against non-crossreacting epitopes carried on the same molecule are suppressed in animals immune just to one of the epitopes. Thus, epitope masking is not a mechanism of IgG-mediated suppression.

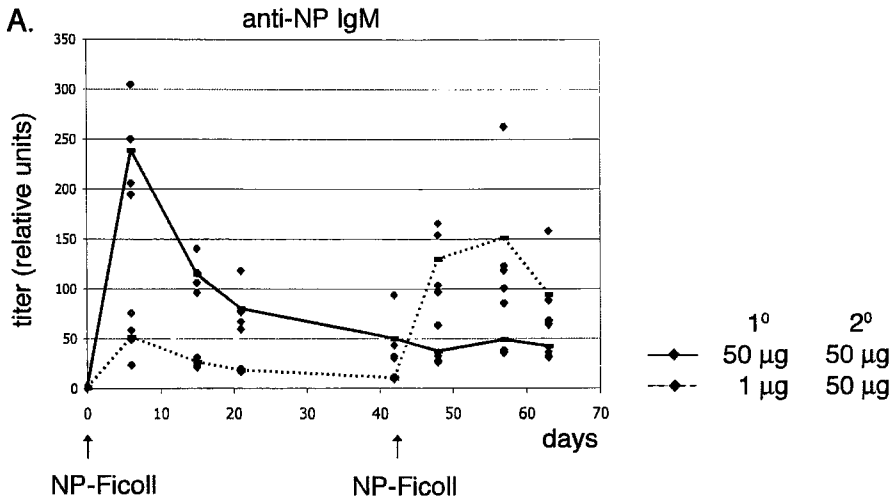
IgG-mediated suppression of T-I type II recall response does not involve known Fcγ receptors

To determine whether IgG-mediated suppression of T-I type II recall responses requires any of the known Fcγ receptors, we tested the recall response to NP-Ficoll in $Fc\gamma RIIB^{-/-}$

and FcRγ$^{-/-}$ mice. Secondary NP-Ficoll immunization did not boost NP-specific antibody titers in these mice (Figures 20A and 20B). Thus, neither FcγRIIB nor any of the FcRγ chain-associated receptors are involved in IgG-mediated suppression of T-I type II recall responses.

Figure 15. Lack of recall response in mice primed with high dose of NP-Ficoll.

Wild-type mice were immunized with 50 or 1 μg of NP-Ficoll and reimmunized with 50 μg of NP-Ficoll six weeks later. NP-specific IgM (A) and IgG (B) titers were measured by ELISA and plotted against the time after primary and secondary immunization. Average values are plotted as bars.


A.
anti-NP IgM
titer (relative units)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
days
NP-Ficoll
NP-Ficoll
1°
2°
50 µg
50 µg
1 µg
50 µg

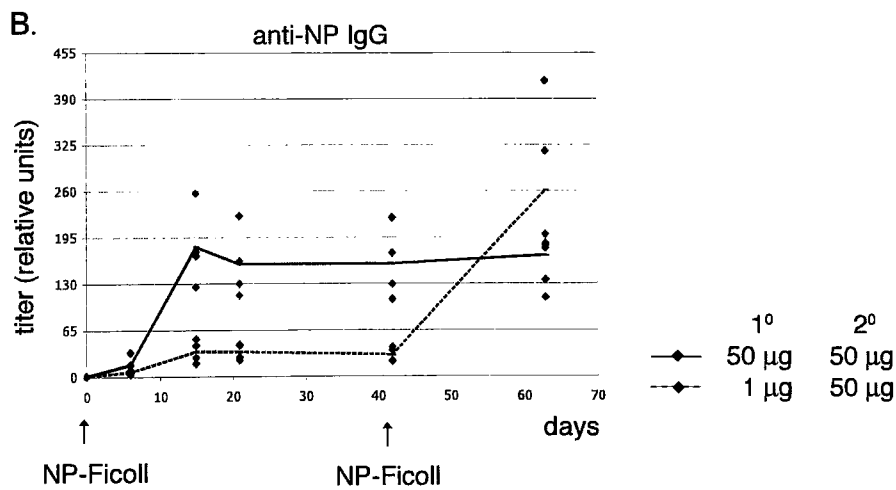
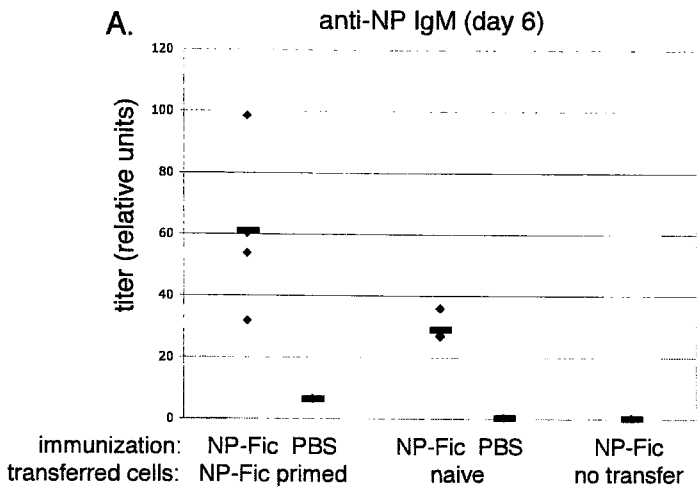

B.
anti-NP IgG
titer (relative units)
455
390
325
260
195
130
65
0
0
10
20
30
40
50
60
70
days
NP-Ficoll
NP-Ficoll
1°
2°
50 µg
50 µg
1 µg
50 µg

Figure 16. Recall response to NP-Ficoll is restored upon adoptive transfer of splenocytes from primed mice into naïve recipients.

Wild-type mice were primed with NP-Ficoll or PBS. On day 15, splenocytes from naïve and primed donors were isolated, depleted of Syndecan-1^{+} plasma cells, and adoptively transferred into naïve PKC$\beta^{-/-}$ recipients, which were immunized with NP-Ficoll or PBS. PKC$\beta^{-/-}$ mice receiving no cell transfer were immunized as a negative control. NP-specific IgM (A) and IgG (B) titers were measured by ELISA on day 6 and 12, respectively.

WT donors --> PKCβ$^{-/-}$ recipients



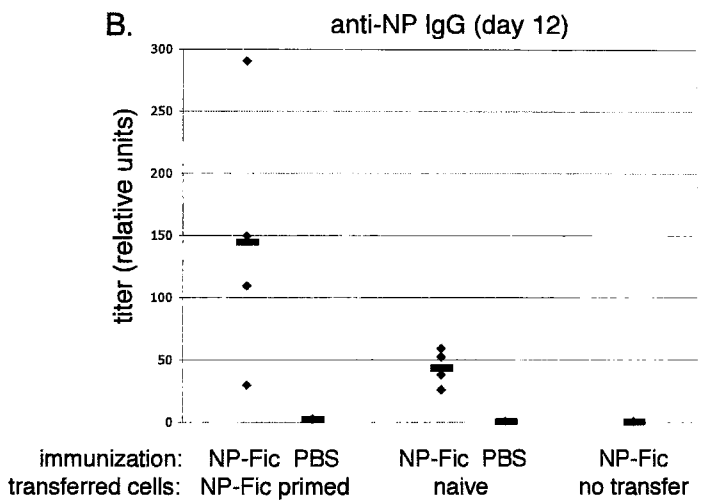
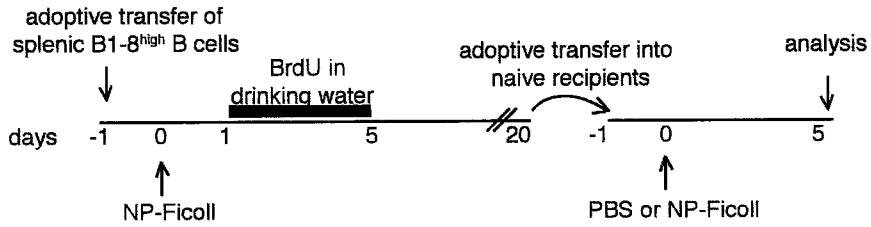


Figure 17. Suppression of T-I memory B cell activation.

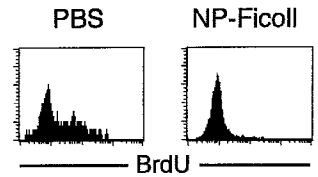
(A and B). Allotype-marked (CD45.1) B1-8^{high} B cells were adoptively transferred into wild-type recipients, which were immunized with NP-Ficoll and fed BrdU (days 1-5). On day 20, primed splenocytes were adoptively transferred into a new group of naïve recipients, which were injected with PBS or immunized with NP-Ficoll. FACS analysis of BrdU loss/retention was done on day 5 after immunization. Gated on $CD45.1^+$ cells.

(C and D). Allotype-marked (CD45.1) B1-8^{high} or $AID^{-/-}$ B1-8^{high} B cells were adoptively transferred into wild-type or $AID^{-/-}$ recipients, respectively, which were immunized with NP-Ficoll and fed BrdU (days 1-5). On day 20, recipients were injected with PBS or re-immunized with NP-Ficoll. FACS analysis of BrdU loss/retention was done on day 5 after secondary immunization. Gated on $CD45.1^+$ cells.

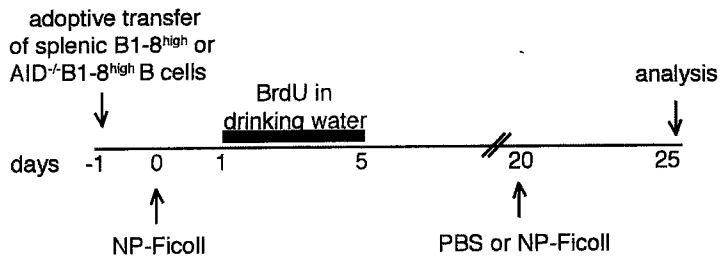
A.


adoptive transfer of
splenic B1-8high B cells
BrdU in
drinking water
adoptive transfer into
naive recipients
analysis
days
-1
0
1
5
20
-1
0
5
NP-Ficoll
PBS or NP-Ficoll

B.


PBS
NP-Ficoll
BrdU

C.


adoptive transfer
of splenic B1-8high or
AID-/-B1-8high B cells
BrdU in
drinking water
analysis
days
-1
0
1
5
20
25
NP-Ficoll
PBS or NP-Ficoll

D.

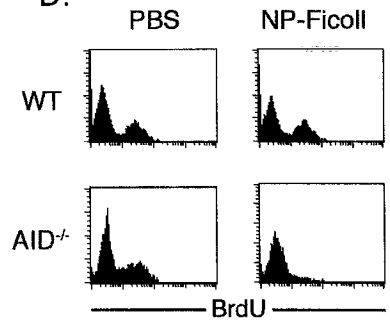
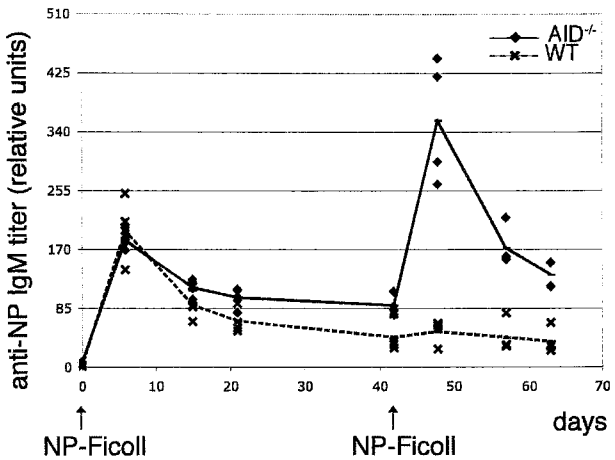

PBS
NP-Ficoll
WT
AID-/-
BrdU

Figure 18. Antigen-specific IgG antibodies account for suppression of recall response to NP-Ficoll.

(A). $AID^{-/-}$ and wild-type mice were immunized with NP-Ficoll and reimmunized six weeks later. NP-specific IgM titers were measured by ELISA and plotted against the time after primary and secondary immunization. Average values are plotted as bars.

(B). NP-specific IgM titers on day 5 after secondary NP-Ficoll immunization of $AID^{-/-}$ mice, pre-injected with NP-specific IgG, non-specific IgG, or PBS. All mice were primed with NP-Ficoll one month before secondary immunization. Background IgM titer values (before secondary immunization) were subtracted. Average values are plotted as bars.

A.


anti-NP IgM titer (relative units)
510
425
340
255
170
85
0
0
10
20
30
40
50
60
70
days
AID-/-
WT
NP-Ficoll
NP-Ficoll

B.

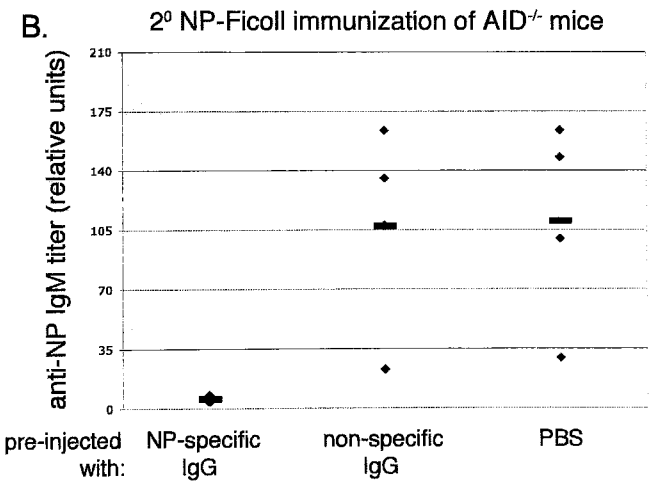
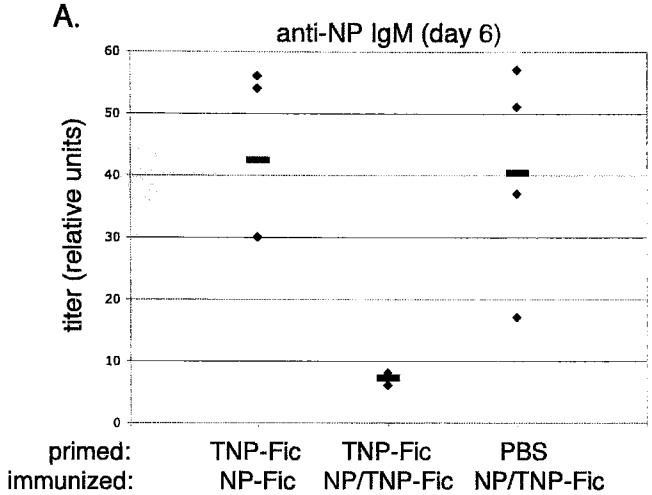

2⁰ NP-Ficoll immunization of AID-/- mice
anti-NP IgM titer (relative units)
210
175
140
105
70
35
0
pre-injected with:
NP-specific IgG
non-specific IgG
PBS

Figure 19. Epitope masking is not a mechanism of IgG-mediated suppression.

Wild-type mice were primed with TNP-Ficoll or PBS two weeks before immunization with NP-Ficoll or doubly-conjugated NP/TNP-Ficoll. NP-specific IgM (A) and IgG (B) titers were measured by ELISA on day 6 and 13, respectively.

A.


anti-NP IgM (day 6)
titer (relative units)
60
50
40
30
20
10
0
primed:
immunized:
TNP-Fic
NP-Fic
TNP-Fic
NP/TNP-Fic
PBS
NP/TNP-Fic

B.

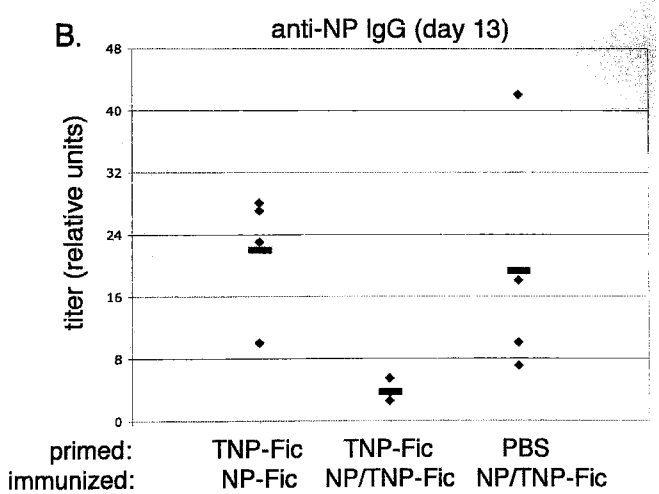
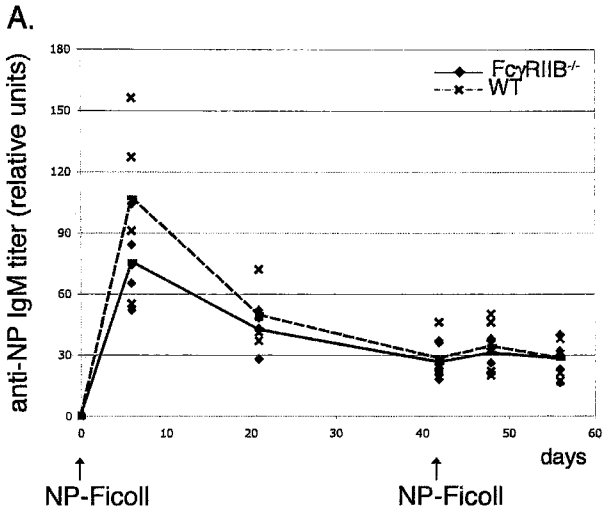
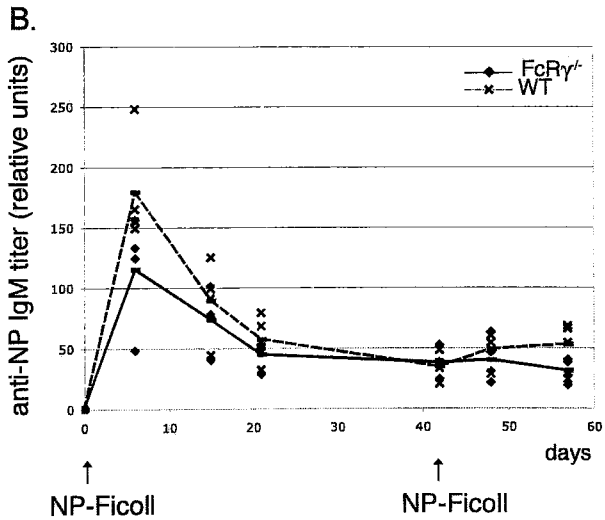

anti-NP IgG (day 13)
titer (relative units)
48
40
32
24
16
8
0
primed:
immunized:
TNP-Fic
NP-Fic
TNP-Fic
NP/TNP-Fic
PBS
NP/TNP-Fic

Figure 20. IgG-mediated suppression of recall response to NP-Ficoll does not involve FcγRIIB or FcRγ chain.

(A). FcγRIIB$^{-/-}$ and wild-type mice were immunized with NP-Ficoll and reimmunized six weeks later. NP-specific IgM titers were measured by ELISA and plotted against the time after primary and secondary immunization. Average values are plotted as bars.

(B). FcRγ$^{-/-}$ and wild-type mice were immunized with NP-Ficoll and reimmunized six weeks later. NP-specific IgM titers were measured by ELISA and plotted against the time after primary and secondary immunization. Average values are plotted as bars.


A.
anti-NP IgM titer (relative units)
FcγRIIB-/-
WT
180
150
120
90
60
30
0
0
10
20
30
40
50
60
days
NP-Ficoll
NP-Ficoll


B.
anti-NP IgM titer (relative units)
FcRγ-/-
WT
300
250
200
150
100
50
0
0
10
20
30
40
50
60
days
NP-Ficoll
NP-Ficoll

DISCUSSION

Characteristics of memory B cells elicited by T-I type II antigens

It has long been debated whether T-I type II responses generate memory B cells. The criterion for B cell memory has been the detection of antigen recall response. Although T-I type II antigens do not elicit recall responses (Hosokawa, 1979; Brodeur and Wortis, 1980; Schuler *et al.*, 1984; Zhang *et al.*, 1988), it has become apparent that this is due to the suppression of T-I type II immune responses by factor(s) present in the immune serum. Primed splenocytes could respond to secondary immunization when adoptively transferred into naïve irradiated wild-type recipients, and injection of the immune serum before the adoptive transfer suppressed this response (Hosokawa, 1979; Brodeur and Wortis, 1980). These findings were consistent with the possibility that T-I type II responses generate memory B cells, although it was unclear whether the transferred cells responding to second immunization in naïve recipients were indeed memory B cells.

We have uncovered memory B cells elicited by polysaccharides and bacteria. Assuming that memory B cells are derived from proliferating antigen-experienced precursors, we used the BrdU pulse-chase strategy for their detection in the B1-8^{high} B cell adoptive transfer system. We show that the majority of BrdU-labeled B1-8^{high} B cells detected in the spleen of the recipient mice after NP-Ficoll immunization have the characteristics of memory B cells. They are quiescent, long-lived cells that do not secrete antibodies and express a B cell marker B220, but not a plasma cell marker Syndecan-1. Furthermore, T cells are not required for the development of B1-8^{high} memory B cells elicited by NP-Ficoll.

Unlike memory B cells that develop after NP-CGG immunization, the majority of T-I memory B cells against NP-Ficoll express low levels of CD21 and CD23. This phenotype distinguishes them from follicular and MZ B cells but not from immature B cells. However, unlike immature B cells, T-I memory B cells are long-lived and contain a small portion of IgG_3^+ cells. Phenotypic resemblance between T-I memory B cells and immature B cells necessitates identification of a T-I memory B cell specific marker to facilitate their analysis in the absence of BrdU pulse-chase.

Our initial identification of T-I memory B cells was done in the B1-8^{high} adoptive transfer system, which relies on high affinity and higher than normal frequencies of antigen-specific B cells (0.2-0.3% of total splenocytes). We therefore needed to confirm that T-I memory B cells exist in mice with a normal (non-transgenic) B cell repertoire. In the absence of a specific T-I memory B cell marker, we identified memory B cells in unmanipulated mice by surface IgG expression and low expression of CD21 and CD23. We found that T cell-deficient mice, capable only of T-I immune responses, contained $B220^+$ IgG^+ B cells with the $CD21^{low}$ $CD23^{low}$ phenotype similar to that of B1-8^{high} T-I memory B cells elicited by NP-Ficoll immunization. In wild type mice, this compartment constituted about one-half of IgG^+ memory B cells. Although this analysis is by necessity restricted to the IgG^+ memory B cells (which constitute about 10% of T-I memory B cells), it validates our findings in the B1-8^{high} adoptive transfer system.

The main route for T-D memory B cell development is the GC reaction (Kocks and Rajewsky, 1989; Rajewsky, 1996; McHeyzer-Williams and McHeyzer-Williams, 2005). However, somatically unmutated memory B cells are elicited by T-D antigens in Bcl-$6^{-/-}$ mice, in which the GC reaction is compromised (Toyama *et al.*, 2002) and in

wild-type mice treated with anti-ICOS antibodies that inhibit the GC reaction (Inamine *et al.*, 2005). These memory B cells might be derived from the extrafollicular response to T-D antigens. It remains to be determined whether T-I memory B cells, identified here, originate from the T-I GC reaction or from the extrafollicular response to T-I type II antigens.

T-I memory B cell longevity and responsiveness to antigens

We have determined that BrdU-labeled T-I memory B cells elicited by NP-Ficoll could be detected up to 4 months after immunization. We wished to compare their lifespan with that of naïve B cells.

The lifespan of naive B cells had been previously estimated using BrdU labeling. The half-life of the splenic B cell compartment, the time point at which 50% of all B cells are labeled with BrdU upon continuous administration, appeared to be 6 weeks in adult mice and 2 weeks in young mice (Förster *et al.*, 1990). This is an underestimate for the half-life of mature naïve B cells, because splenic B cells also contain short-lived immature B cells, whose half-life is about 4 days (Allman *et al.*, 1993). To estimate the half-life of the naïve B cell compartment, we adoptively transferred allotype-marked splenic B1-8^{high} B cells and followed their survival in wild-type recipients. Our estimate of the half-life of naïve B cells turned out to be approximately 90 days. The rate of reduction of T-I memory B cells was similar to that of naïve B1-8^{high} B cells, suggesting that naïve and T-I memory B cells have a similar lifespan.

We next compared the proliferative response of B1-8^{high} B cells from NP-Ficoll primed and naïve mice to a range of NP-Ficoll doses after their adoptive transfer into

naïve recipients. We found no difference in the sensitivity of B1-8^{high} naïve and T-I memory B cells to the lowest dose tested (1 μg). This approach, however, has inherent caveats. First, adoptive transfer of B cells results in a low degree of non-specific proliferation, obvious by a diffuse CFSE peak in unimmunized controls (Figure 6B). This makes it unfeasible to test NP-Ficoll doses lower than 1 μg. Second, both primed and naïve B cells used in this experiment were of high affinity to antigen, which may not reflect a physiologic scenario. Third, not all Igλ^{+} B1-8^{high} B cells from primed mice might have been memory B cells. Fourth, although splenic localization of T-I memory B cells has not been yet determined, it is possible that adoptive transfer disrupts their proper localization, which might give them an advantage in antigen contact.

Although we cannot conclude that T-I memory B cells are more sensitive to T-I type II antigens than naïve B cells of the same affinity, T-I memory B cells might be more sensitive than naïve B cells to stimulation by T-D antigens. This is suggested by the studies, in which NP-Ficoll primed mice responded to a very low dose of NP-CGG provided without any adjuvant, whereas naïve mice did not (Maizels *et al.*, 1988). Furthermore, human memory B cells are uniquely responsive to T-I type I stimuli *ex vivo* (Bernasconi *et al.*, 2002), although T-I versus T-D origin of human memory B cells used in those experiments was not known.

T-I memory B cell selection based on BCR affinity

Burnet's theory predicts selection of clones of higher affinity to antigen during the immune response. Based on the competition assay between adoptively transferred B1-8^{high} and B1-8^{low} B cells with a 40-fold difference in affinity to NP, Shih *et al.* suggested

that this selection occurs at the stage of B cell activation in response to NP-Ficoll (Shih *et al.*, 2002). However, in contrast to the dominance of the V_H186.2 heavy chain-expressing clones after NP-CGG immunization (Cumano and Rajewsky, 1985), NP-Ficoll immunization results in a heterogeneous collection of NP-specific hybridoma clones (Maizels and Bothwell, 1985), reflecting lack of selection at the stage of B cell activation by NP-Ficoll in wild-type mice.

Here we tested the effect of BCR affinity on the development of T-I memory B cells. B1-8^{low} B cells, adoptively transferred into wild-type recipients, did not develop the T-I memory B cell compartment, although they responded to NP-Ficoll immunization and formed both T-I GCs and plasma cells. However, B1-8^{low} B cells were capable of forming the memory compartment in PKC$\beta^{-/-}$ recipients, whose own B cells do not respond productively to T-I type II antigens (Leitges *et al.*, 1996). Our data suggest that BCR affinity-based competition occurs during the establishment of the T-I memory B cell compartment rather than at the stage of B cell activation and are in agreement with findings by Maizels and Bothwell mentioned above.

Analysis of hybridomas derived from wild-type mice revealed that re-immunization of NP-Ficoll primed mice resulted in a different composition of the NP-specific hybridoma repertoire compared with that after primary NP-Ficoll immunization. Remarkably, clones in the secondary repertoire were of higher affinity than in the primary repertoire, but did not contain somatic mutations (Maizels *et al.*, 1988). These data are consistent with our findings that B1-8^{low} clones are excluded from the long-lived T-I memory B cell compartment in the competitive environment of wild-type recipients, which would result in the repertoire shift towards higher affinity clones in the absence of

somatic mutation. It remains to be determined how the selection of higher affinity clones in the T-I memory B cell compartment is enforced at the molecular level.

Our data implicate BCR-antigen interaction in the establishment or the survival of the long-lived T-I memory B cell compartment. It has been reported, however, that memory B cells derived from T-D responses do not require immunizing antigen for survival (Maruyama *et al.*, 2000). A different outcome of our studies may reflect the fact that we focused on an early stage of memory B cell development, whereas Maruyama *et al.* tested antigen requirement for memory B cell survival starting 6-8 weeks after immunization. It cannot be ruled out, however, that T-I and T-D memory B cells differ in their requirement for interaction with an immunizing antigen.

The role of Blimp-1 in T-I type II B cell response

Blimp-1 is a transcription factor necessary for antibody production. T-D responses have been thoroughly analyzed in Blimp-1 knock-out mice, which lack plasma cell formation and generate enlarged GCs (Shapiro-Shelef *et al.*, 2003). It has been speculated that oversized GCs are due to the accumulation of cells whose differentiation to plasma cells is blocked in the absence of Blimp-1 (McHeyzer-Williams and McHeyzer-Williams, 2005).

Here we examined the role of Blimp-1 in the T-I type II B cell response. We found that conditional disruption of Blimp-1 in B cells did not affect B cell activation in response to NP-Ficoll immunization, but caused a significant reduction in the number of plasma cells and an increase in the number of GC B cells, which is analogous to the effect of Blimp-1 disruption on the T-D response. Since plasma cell formation precedes

the GC formation during T-I type II responses, our findings suggest that Blimp-1 has two independent functions in B cells: allowing plasma cell development and limiting the GC reaction.

In our experiments Blimp-1 deficiency resulted in a lower number of T-I memory B cells detected by the BrdU pulse-chase strategy. It is unclear whether Blimp-1-deficient B cells simply proliferate longer than wild-type B cells and thus lose the BrdU label or whether Blimp-1 deficiency genuinely affects T-I memory B cell development or survival. T-I memory B cells do not express Blimp-1 and are not derived from Blimp-1 expressing precursors. Thus, Blimp-1 is unlikely to play a direct role in T-I memory B cell development.

It is possible that Blimp-1 requirement for T-I memory B cell development, if any, is indirect—i.e., due to the absence of antibodies or plasma cell-derived cytokines in Blimp-1-deficient mice. Interestingly, $B220^-$ T-D memory B cells, which do not express Blimp-1 (McHeyzer-Williams and McHeyzer-Williams, 2005), are absent in Blimp-1-deficient mice (Shapiro-Shelef *et al.*, 2003) and thus may have a similar indirect requirement of Blimp-1 for their development or survival. The involvement of antibodies of secondary isotypes in T-I memory B cell formation can be ruled out, since we were able to detect BrdU-labeled T-I memory B cells in $AID^{-/-}$ mice, deficient in class switch recombination (Muramatsu *et al.*, 2000). It remains to be tested whether IgM antibodies are required for the development of T-I and $B220^-$ T-D memory B cells, and if so, whether they act through complement fixation and the engagement of complement receptors or through the engagement of Fcα/μR.

IgG-mediated suppression

T-I memory B cells do not respond to secondary immunization with T-I type II antigens in the presence of antigen-specific IgG antibodies generated during the primary immune response. Similarly, naïve B cells do not respond to T-I type II antigens in the presence of passively transferred antigen-specific IgG antibodies. The presence of IgG, however, does not preclude reactivation of memory B cells by T-D or T-I type I antigens (Hosokawa, 1979; Zhang *et al.*, 1988), suggesting that the properties of T-I type II antigens make them the target for IgG-mediated suppression.

Although the phenomenon of IgG-mediated suppression of primary responses was known before, the physiologic relevance of regulation of primary B cell responses by passively transferred immune serum was uncertain, because antigen-specific antibodies normally appear only after a productive B cell response. Our findings underscore a physiologic regulatory function for IgG-mediated suppression. Due to their poor biodegradability, T-I type II antigens, including synthetic and native bacterial polysaccharides, are retained in the organism for long periods (Humphrey, 1981). The existence of an expanded and long-lived pool of memory B cells capable of responding to such persistent antigens necessitates a suppressive mechanism to prevent their continuous reactivation leading to antibody overproduction. Antigen-specific IgG, a product of the immune response, serves this important negative feedback regulation, thereby maintaining humoral homeostasis.

The mechanism of IgG-mediated suppression has not been yet elucidated. It has been reported that anti-NP antibodies suppress the response both against NP and its carrier, sheep red blood cells, but not against co-injected unhaptenated horse red blood

cells (Brüggemann and Rajewsky, 1982). Although this observation could rule out a hypothesis that IgG-mediated suppression is due to epitope masking, it was argued that haptens might block the access of B cells to the carrier (Heyman, 1993). Here we demonstrate that when two non-crossreacting epitopes (NP and TNP) are coupled to the same T-I type II carrier molecule (Ficoll), and thus should be equally accessible to B cells, antibodies specific to one of the epitopes suppress the immune response against both. It is therefore likely that an Fc portion of IgG antibodies is involved in suppression. However, known Fcγ receptors do not appear to be involved in the suppression of primary (Karlsson *et al.*, 1999; Karlsson *et al.*, 2001) or secondary T-I type II responses.

Implications for polysaccharide vaccines

We show that T-I type II responses, induced by a model polysaccharide antigen NP-Ficoll or bacteria, generate memory B cells. Several polysaccharide vaccines have been developed, e.g. Pneumovax and Menomune, which combine purified capsule polysaccharides from a number of *S. pneumoniae* and *N. meningitidis* strains, respectively, to induce long-term humoral protection against these pathogens in adult humans. Based on our findings in mice, we speculate that polysaccharide vaccines may elicit memory B cells in humans. It remains to be tested to what extent memory B cells contribute to long-term antibody production against poorly metabolized polysaccharide antigens under the regulation of IgG-mediated suppression, and whether by slowly replenishing the plasma cell pool, T-I memory B cells extend the effectiveness of polysaccharide vaccines.

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