Dynamics of Acquisition of Antigen and T Cell Help Influence B Cell Fate in the T-Dependent Humoral Immune Response

by

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ABSTRACT

The T-dependent humoral immune response is critical for generation of durable high-affinity antibody responses and protection from many pathogenic infections. B cell recruitment into the T-dependent humoral immune response requires acquisition of both antigen (Ag) and T cell help, and reacquisition of Ag and T cell help is thought to promote B cell proliferation, germinal center (GC) recruitment and selection, and differentiation into memory B cells and plasma cells (PCs). Despite the central role of Ag and T cell help in driving humoral immune responses, how the dynamics of acquisition of Ag and T cell help by B cells affect their participation in different stages of the T-dependent humoral immune responses is poorly understood.

During the initial stages of the humoral immune response B cell access to Ag may be limited, and intravital imaging studies suggest that in some cases B cells' early encounters with Ag may be transient. Whether B cells are tolerized or can be recruited into humoral immune responses following such encounters is not clear. We found that in contrast to B cells that continuously acquire Ag but not T cell help, B cells transiently exposed to Ag did not become anergic or undergo apoptosis in the absence of T cell help; rather they returned to a naïve-like state and were able to participate in subsequent immune responses. When T cell help was available, single transient Ag acquisition enabled B cell proliferation and differentiation into PCs and various subpopulations of memory B cells. Transient Ag acquisition also enabled B cells' recruitment into GCs, even during the contraction phase of the GC response, in contrast to newly arriving B cells not preloaded with Ag, which were severely limited in their ability to participate in GCs after the peak of the GC response. Altogether these results indicate that when T cell help is available, transient Ag acquisition enables efficient recruitment of B cells into the T dependent humoral immune response, and favors B cell survival in the absence of T cell help.

Within GCs, B cells compete for acquisition of Ag and T cell help following random mutation of their BCRs. Those that compete successfully are positively selected, and undergo further rounds of mutation and selection or differentiate into high-affinity PCs or memory B cells. The precise mechanisms of selection are still unclear and the individual roles of Ag

acquisition and T cell help are an area of active investigation. While recent studies have provided evidence that competition for T cell help can drive selection of GC B cells, whether Ag acquisition can also support GC B cell selection independently of its role in promoting acquisition of T cell help is not known. We addressed the roles of Ag acquisition and T cell help separately and in combination and found that BCR cross-linking alone is not sufficient to promote GC B cell selection. Preliminary results suggest that BCR cross-linking is not necessary to promote T cell help based selection, but that it may enhance GC B cells' expansion and differentiation into PCs.

CHAPTER 1. INTRODUCTION

Importance of humoral immunity

The importance of the humoral immune response has been appreciated for over a century. In the late 19th Century it was recognized that humour (serum) from an individual immune to a given pathogen could be transferred to a susceptible one to confer protection, and this procedure became one of the first forms of immunotherapy (1). It was later discovered that antibodies, also called immunoglobulins (Igs), were the therapeutic component of immune serum and that they were derived from B lymphocytes (2, 3). Major advances in understanding the B cell-mediated immune response have been made, but transfer of pre-formed antibodies to patients continues to be a widely used and highly effective immunotherapy, and has expanded in scope beyond conferring protection against pathogens; current applications range from antivenom to cancer therapy to treatment of autoimmune diseases to recently developed anti-ebola antibodies (4, 5). The humoral immune response also forms the basis of almost all successful vaccines, which represent one of the most effective public health advancements in human history, drastically reducing the incidence of a number of devastating diseases. Vaccination is based on the introduction of **anti**body-**gen**erating substances (antigens, Ags) derived from the pathogen against which the vaccine is targeted in a non-pathogenic context, enabling production of Ag-specific antibodies while minimizing the risks of pathogenic effects associated with infection (6). In addition to helping fight pathogens, antibodies themselves can act in a pathogenic manner when they recognize Ag produced by the individual in which they were generated. Such self-reactive antibodies are a major contributor to pathogenesis of various autoimmune diseases and represent a breach in the normal enforcement of tolerance to self (7).

Antibody diversity and tolerance

The roles of the humoral immune response in pathogen clearance and autoimmunity are predominantly mediated through antibodies. One of the factors that make the humoral immune response so potent is that antibodies can be generated that recognize virtually any molecular structure. This diversity is partially encoded at the beginning of B cell development, when B

cells developing in the bone marrow recombine gene segments to encode a B cell receptor (BCR) with a single specificity, a membrane-anchored antibody that recognizes Ag and transmits signals to the B cell. The number of gene segments encoding the variable Ag recognition regions of BCRs enables approximately 2.6 million unique combinations, but additional diversity encoded by random insertion and deletion of nucleotides between the gene segments during rearrangement enables generation of BCRs capable of recognizing virtually any molecular structure (8).

The diversity of the BCR repertoire ensures that some of the BCRs are reactive against self Ag. Developing B cells that rearrange self-reactive BCRs undergo additional variable gene segment rearrangement to alter their specificity, and cells that continue to bind self Ag with high avidity in the bone marrow die. Immature B cells that successfully rearrange their BCRs and do not bind self Ag leave the bone marrow and migrate to secondary lymphoid organs (SLOs), the spleen and lymph nodes (LNs), where they enter follicles and complete maturation. Immature B cells that bind Ag in the periphery prior to maturation are silenced through deletion or induction of an unresponsive state termed anergy that will be discussed in detail below. Mature naïve B cells circulate through the blood and lymphatic circulatory systems, where they can encounter cognate Ag.

Anatomy of SLOs

The primary site of Ag recognition by naïve B cells is in SLOs, including LNs, spleen, and Peyer's patches, to which Ags drain or are delivered by immune cells from the local interstitial space of the skin or mucosa, blood, and gut, respectively (9). SLOs are organized into discrete compartments in which B cells, T cells, and other immune cells are organized. This spatial organization is defined by various kinds of resident stromal cells that produce distinct chemokines and other factors, which are differentially recognized by lymphocytes and other migrating cells through specialized G protein-coupled receptors. The chemokines CCL19 and CCL21 are produced by the stromal cells in the T cell zone and attract T cells and dendritic cells (DCs) through the receptor CCR7, while follicular stromal cells produce the chemokine CXCL13, which recruits B cells via its receptor CXCR5 (refs. 10, 11). B cell follicles are located adjacent to the subcapsular sinus, the outer space through which afferent draining lymph enters the LN, while the T zone borders the follicles from the medullary side. Lymphocyte areas are

organized similarly in the splenic white pulp, with T cells localized to the periateriolar lymphoid sheath (PALS) surrounding arterioles, B cell follicles adjacent to the PALS, and both surrounded by the marginal zone, to which blood-borne Ags drain. Naïve B and T cells enter SLOs predominantly from the blood or upstream lymphatics. They then travel along the CXCL13 and CCL19/21 gradients to their respective zones where they continue active migration in search of cognate Ags. Naïve lymphocytes that do not encounter Ag in the SLO leave after approximately 6-24h and return to blood circulation either directly through the MZ of the spleen or indirectly via the cortical and medullary lymphatic sinuses of LNs and Peyer's patches, through the efferent lymphatics, and then large thoracic duct (12-14). However, if they encounter cognate Ag during their migration in SLO, they may undergo activation, be temporarily prevented from returning into lymph/blood circulation, and participate an immune response.

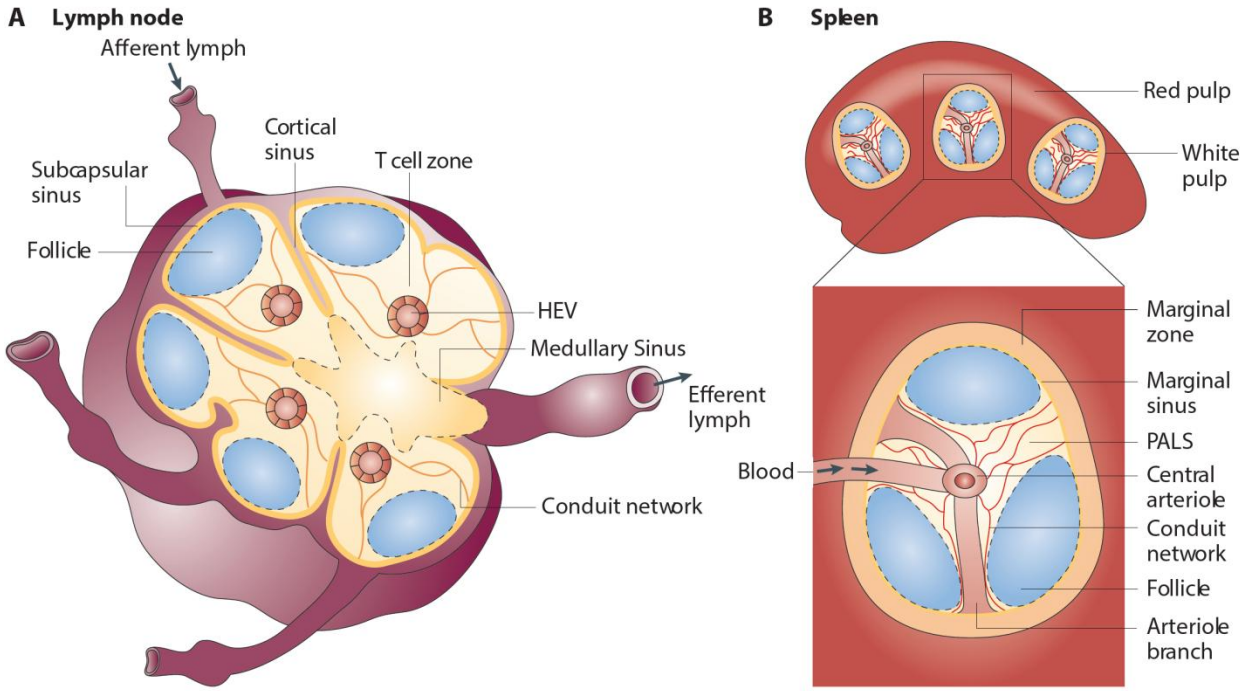

Figure 1.1. Anatomy of lymph nodes and spleen. Adapted from Batista, F. D. and N. E. Harwood, *Nat. Rev. Immunol.* 9:15-27 (2009) (ref. 15). Architechture of (**A**) LNs and (**B**) spleen.

BCR signaling

When B cells encounter cognate Ag, its binding to the BCR initiates BCR signaling. The BCRs expressed by naïve B cells do not have their own signaling domains, but associate with a

heterodimer of the transmembrane proteins Igα and Igβ, which have intracellular tails containing immunoreceptor-tyrosine based activating motifs (ITAMs). Ag binding induces phosphorylation of the ITAMs on Igα-Igβ by the Src family kinases Lyn, Blk, and Fyn, and recruitment and activation of spleen tyrosine kinase (Syk) which phosphorylates the adaptor protein B-cell linker (BLNK). Phospho-BLNK serves as a scaffold for assembly of a group of signaling enzymes, including Bruton's tyrosine kinase (Btk) and phospholipase C-gamma 2 (PLCγ2). Syk and Btk activate PLCγ2, and Syk also activates phosphoinositide 3-kinase (PI3K) through B-cell adaptor for PI3K (BCAP). PLCγ2 and PI3K generate secondary lipid messengers that initiate multiple signaling pathways through the downstream kinases AKT and protein kinase c (PKC), calcium signaling, and activation of Ras which can lead to changes in cell metabolism, gene expression, and cytoskeletal organization (16-18). BCR signaling also induces clustering and endocytosis of the Ag-bound BCRs, along with the Ag. The endocytosed BCRs continue to signal after internalization, and BCR endocytosis is necessary for proper regulation of BCR signaling and transcriptional activation (19). Interestingly, tonic signaling through the BCR and its activation of PI3K is necessary for B cell survival (20, 21). B cell survival is also dependent on B cell activating factor of the tumor necrosis factor family (BAFF), secreted by follicular stroma and other cells. BAFF signaling through its primary receptor on B cells, BAFFR, has been demonstrated to utilize Syk phosphorylation through Igα-Igβ (22).

Ag-triggered BCR signaling initiates a signaling cascade that can activate the transcription factor complex AP1 through the Ras/MAPK cascade and NFκB and NFAT through the second messengers generated by PLCγ2 and PI3K (17, 18). These transcription factors activated by Ag-induced BCR signaling lead to a number of transcriptional changes in the cell that prime it to receive additional signals that prompt proliferation and differentiation.

Secondary diversification and effector B cells

Depending on the signals they receive, activated B cells may alter their BCRs to enhance their responsiveness against a pathogen. During affinity maturation, mutations are made in the gene encoding the Ag-binding regions of the BCR, and mutations that enhance Ag binding are selected. Affinity maturation takes place within germinal centers (GCs), and will be discussed in more detail in the following sections.

In addition to altering Ag-binding sites, activated B cells given appropriate signals can make changes to the constant region of their BCR. The constant region is anchored in the membrane and associates with the Igα-Igβ heterodimer to transmit signals, and when BCRs are secreted as antibodies the constant region is recognized by other cells of the immune system. Exchanging the constant region for a different isotype can therefore alter BCR signaling and antibody function, but does not change the specificity for Ag. Naïve B cells co-express two isotypes of BCR, IgM and IgD; these can be exchanged for IgE, one of two subclasses of IgA, or one of 4 subclasses of IgG in humans. Each antibody isotype interacts with cells and fixes complement differently and is tailored to enhance immune responses against different types of pathogens. Secondary diversification of BCRs is mediated by activation-induced cytidine deaminase (AID), which introduces point mutations in variable region genes during affinity maturation and double stranded-breaks in constant region genes during isotype switching. B cells that have undergone affinity maturation or have switched isotype differentiate into effector B cells, of which there are two types, plasma cells (PCs) and memory B cells. However, secondary diversification is not required for effector differentiation; many memory and PCs express IgM and have not undergone affinity maturation.

PCs are large terminally differentiated B cells, so named for their large amount of cytoplasm, which houses robust protein synthesis and export machinery, enabling continual production and secretion of antibodies (23, 24). PCs do not divide, but some can survive, constantly secreting antibodies, for many years (25). Plasmablasts (PBs) are an intermediate between activated B cells and terminally differentiated PCs; they secrete antibodies and are capable of proliferating, but are not long-lived; they either differentiate into PCs or die (26, 27).

Memory B cells are Ag-experienced cells that are quiescent and like naïve B cells, they circulate through the blood and lymph. Upon re-encounter with cognate Ag, they are more readily activated than their naïve counterparts, and they can help activate other cells of the immune system and rapidly generate PCs (28, 29). Differentiation of memory and PCs requires Ag-activated B cells to integrate signals from many different sources, and these signals can differ depending on the context in which Ag is encountered as well as properties of the Ag itself. Differentiation of effector B cells will be discussed in more detail as B cell responses to Ag in different contexts are described in the following sections.

T-independent and T-dependent B cell responses

Bretcher and Cohn's two-signal hypothesis of lymphocyte activation predicts, and experimental evidence bears out, that Ag receptor signaling alone is insufficient for full activation of lymphocytes and their differentiation into effector cells (30, 31). The "second signal" required for B cell effector differentiation is likely to be multiple signals in most physiological settings, and these can be acquired from different sources. Well before Bretcher and Cohn's hypothesis, experiments were performed that separated antibody responses into two broad categories, those that could be generated in thymectomized mice and those which could not, and hence were described as T-independent and T-dependent responses, respectively. T-independent responses can be generated by Ags that deliver strong activating signals, and fall into two categories, T-I type 1 and T-I type 2. T-I type 1 refers to B cell responses to Ags that promote B cell proliferation independently of BCR signaling; these Ags are therefore considered mitogens, and at high concentrations can induce widespread B cell proliferation and PB differentiation, resulting in non-Ag specific antibody production. At lower concentrations, however they can induce more specific antibody responses due to their selective acquisition by and consequent stimulation of cognate B cells. T-I type 2 Ags engage B cells through their BCRs and tend to be highly cross-linking; their repetitive BCR binding sites induce very strong BCR signaling. Examples of T-I type 2 Ags include bacterial polysaccharides and viral capsids. Co-stimulation through BAFFR can enhance T-independent responses and promote isotype switching when B cells acquire these Ags from activated innate immune cells such as dendritic cells (DCs) and macrophages; they can also be enhanced by complement and microbial pattern recognition receptor signaling (32-34). In general, T-independent responses lead to low affinity short-term humoral immunity. However, in some cases they may generate long-lived antibody and memory responses (28, 35-37).

In contrast to T-independent responses, T-dependent humoral immune responses efficiently generate long-lived PCs and memory cells, enhancing protection against previously encountered pathogens. The T-dependent response is unique in that it can adaptively enhance affinity and specificity to Ag beyond that encoded during development through affinity maturation, contributing to its essential role in generating long-lived protective immunity. Paradoxically, T-dependent responses can be generated against less inherently stimulating Ag than is required for T-I responses. More efficient production of long lived memory and PCs and

generation of higher-affinity antibodies is elicited in T-dependent responses because the "second signal" comes from activated helper T (Th) cells, which can provide particularly potent B cell stimulating factors. Therefore activation of both Ag-specific B and Th cells is required for a successful T-dependent humoral response.

Anatomy of the early T-dependent B cell response

On the B cell side of the response, activation is initiated when B cells recognize and acquire cognate Ag, triggering BCR signaling and endocytosis of Ag-bound BCRs (**Fig. 1.2A**, **i**). BCR signaling initiates a complex sequence of changes in surface expression levels of various G protein-coupled receptors that drive B cell migration to various regions of the SLO at specific times (11). Within approximately 6 hours, activated B cells upregulate the chemokine receptor CCR7, promoting their migration towards the CCL19 and CCL21-rich T cell zone (38). Their uniform distribution at the T-B border is also promoted by upregulation of the oxysterol receptor Epstein-Barr virus induced protein 2 (EBI2), which recognizes 7α,25-dihydroxycholesterol, located in higher concentrations at the follicular perimeter than in the center (39-42). Additionally, activated B cells upregulate the costimulatory molecule CD86 and present proteolysed antigenic peptides loaded in MHCII for recognition by cognate Th cells (43, 44). At the border of the follicle, the activated B cells exchange activating signals with cognate Th cells (45, 46) (**Fig. 1.2A**, **ii**). After acquisition of T cell help B cells further upregulate EBI2 and decrease expression of CCR7, promoting their redistribution to interfollicular regions, where they undergo proliferation (**Fig. 1.2A**, **iii**). After proliferating, they commit to memory, PC, or germinal center (GC) (11, 46). (**Fig. 1.2B**, **iv**, **v**). The signals received from Th cells can affect differentiation and the extent of proliferation of the activated B cells and include CD40L and the cytokine IL-21, which enhance proliferation and PC differentiation; production of IL-4 can promote isotype switching to IgG_1 (refs. 47, 48-50). Usually, early memory cells are generated approximately 2-5 days after B cells acquire Ag and are relatively low-affinity and predominantly non isotype-switched (51, 52). Similarly, the first PBs are generated approximately 2-4 days after B cells acquire Ag, and produce an early wave of relatively low-affinity, predominantly unswitched antibodies. T cell help is thought to promote proliferation of these early PBs, leading to predominance of those with the highest affinity for Ag, which can acquire more Ag to present to Th cells and therefore outcompete their lower affinity counterparts

(53, 54). A similar mechanism governs B cell recruitment into GCs; while low-affinity B cells can be recruited into GCs, competition for T cell help promotes increased recruitment of clones with higher affinity to Ag (54, 55). Starting 3-4 days after B cells acquire Ag, GC precursors downregulate EBI2 and move from the edge of the follicle into its center, following a specialized population of Th cells known as T follicular helper cells (Tfh), described below (46, 56). Within the GCs, B cells proliferate extensively, undergo isotype switching, and undergo iterative rounds of random mutation of their BCRs and selection of the highest affinity mutants based on competition for Ag and Tfh help (57). Positively selected GC B cells differentiate into high-affinity memory and long-lived PCs and are critical for durable protective humoral immunity (**Fig 1.2B**, **vi**).

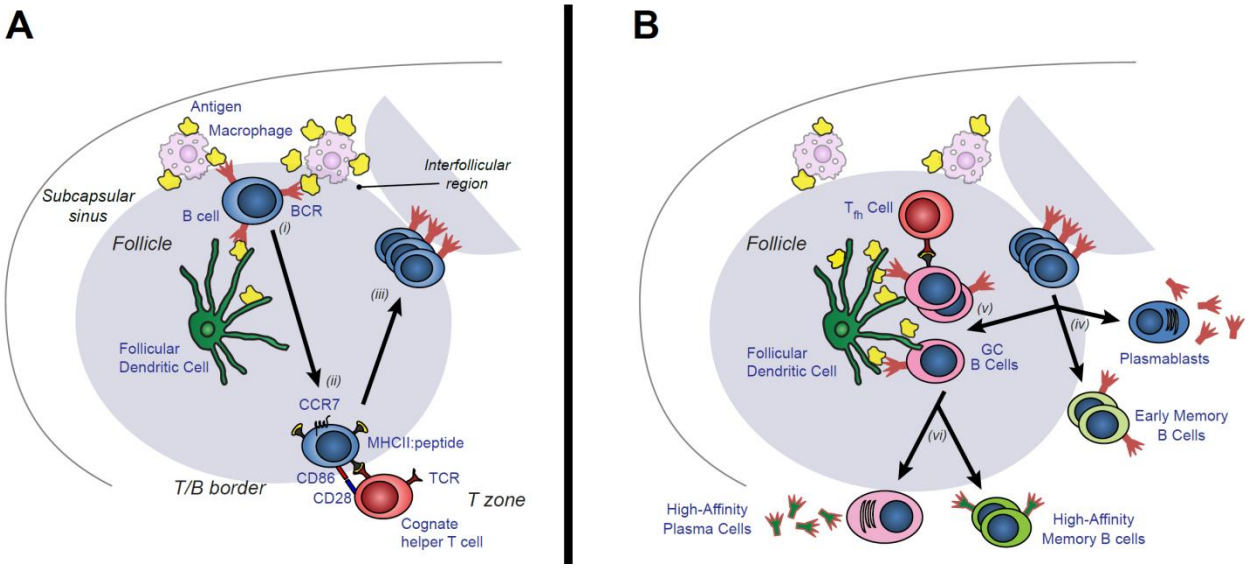

Figure 1.2. Outline of the T-dependent humoral response.

Follicular helper T cells

On the T cell side of the T-dependent humoral response, activation is initiated when Ag-specific Th cells recognize cognate Ag with their T cell receptors (TCRs) in the form of antigenic peptides loaded in MHCII and presented by professional Ag presenting cells (APCs), such as DCs. Similarly to B cells, TCR binding to Ag initiates an intracellular signaling cascade resulting in transcriptional changes in the T cell. T cells also require a "second signal" for productive activation, usually delivered in the form of co-stimulatory molecules upreguated by activated APCs such as CD86, which binds to CD28 on T cells (58). Strong Ag priming and

costimulation promote initial upregulation of the transcriptional repressor B cell lymphoma 6 (Bcl6), which promotes and is critical for Tfh differentiation (59). Expression of Bcl6 can be reinforced by interactions with professional Ag presenting cells expressing inducible costimulator (ICOS) ligand (ICOSL), which engages CD28 family member ICOS on T cells (60-62). Production of the cytokines IL-6 and IL-21 by activated APCs may also support early Bcl6 expression (63, 64). Bcl6 expression and Tfh differentiation can be diverted by exposure to cytokines promoting alternate Th subset differentiation pathways, as well as the canonical T cell proliferation cytokine IL-2, which upregulates the transcriptional repressor B lymphocyte induced maturation protein 1 (Blimp1). Blimp1 and Bcl6 are mutually suppressive, limiting Tfh differentiation to those cells in which stabilization of Bcl6 can overcome Blimp1 or diversion to other Th effector types (59, 61, 62, 65). Activated Th migration towards the follicle may be promoted by a decrease in CCR7 shortly after activation, and Bcl6 can also promote CCR7 downregulation and CXCR5 upregulation in pre-Tfh cells, enhancing their migration towards the B cell follicles, near which they form long contacts with activated cognate helper B cells within approximately 1-2 days following Ag stimulation (45, 46, 61, 66). Interactions between signaling lymphocytic activation molecule (SLAM) on activated B cells and SLAM-associated protein (SAP) on T cells are necessary for these long contacts, and disrupting them leads to a defect in Tfh differentiation and blocks GC formation (67). In addition to providing activating signals to B cells, pre-Tfh cells receive activating signals that promote Tfh differentiation during these long contacts, including through CD80/CD28 and ICOSL/ICOS interactions (65, 68). After approximately 2 days, Tfh cells with characteristically high expression of CXCR5 and the CD28 family member programmed death 1 (PD-1) downregulate EBI2 and migrate into the follicle, where they form GCs with GC B cells (66, 69). Tfh cells are critical for maintenance of GCs, in which they interact with Ag-presenting GC B cells, forming brief but extensive contacts and providing pro-survival signals to those presenting the most Ag, facilitating selection of high affinity GC B cells. These extensive contacts require interaction between ICOS and ICOSL (70, 71). Some of the factors Tfh cells provide to GC B cells during these contacts include CD40 signaling and the cytokines IL-21, IL-4, and IFN- γ; these are all important for promoting GC B cell proliferation, isotype switching, and influencing memory and PC differentiation, and will be discussed below (72-75).

Follicular regulatory T cells (Tfr cells)

A second subset of follicular T cells, called Tfr cells, is important for proper control of the Tfh and GC B cell responses. Tfr cells share a dependence on Bcl6 for differentiation and high expression of CXCR5 and PD-1 with Tfh, but they also express Blimp1 and the regulatory T cell (Treg) transcription factor FoxP3, and they negatively regulate humoral responses. Tfr cells differentiate predominantly from natural Tregs, which differentiate during development when they bind self-Ag, although Tfr cells have been shown to differentiate from foreign Ag-specific Th cells under certain immunization conditions (76-78). Like Tfh, Tfr differentiation requires strong co-stimulatory signals through CD28 and ICOS, but signaling through PD-1 may selectively inhibit their differentiation and activity compared to Tfh (77, 79, 80). Tfr cells limit the overall size of GCs as well as expansion of non Ag specific GC B cells, and increases in both high and low affinity antibodies have been observed when they are deleted or their differentiation is blocked (76, 77). *Ex vivo* cultures of Tfr cells with Tfh and GC B cells have demonstrated Tfr can suppress isotype switching and antibody production by GC B cells and IL-21 and IL-4 secretion by Tfh cells *in vivo* (81). Expression of CTLA4, the inhibitory high-affinity receptor for CD80 and CD86, by Tfr cells has been demonstrated to be important for this regulation, and may also support selection of high-affinity GC B cells, as lower-affinity antibody responses were observed when CTLA4 was deleted on Tfr cells (82). Together Tfh and Tfr cells promote and control the magnitude and specificity of GC response and proper differentiation of effector cells.

Spatio-temporal dynamics of Ag acquisition by B and T cells *in vivo*.

Recruitment of B cell clones into T-dependent response is likely to depend on the timing of Ag acquisition by B and cognate Th cells. Therefore, spatio-temporal dynamics of Ag trafficking to SLOs can affect these critical first steps. The availability of Ag to B cells depends on a number of factors, including size, its route of entry, and availability of preexisting Ag-specific Abs. Smaller Ags, below 70 kDa, such as bacterial toxins or proteolyzed pathogen fragments can drain through gaps in the floor of the SCS or enter follicles through conduits, where they can be readily acquired by Ag specific B cells (43, 44). In contrast to small Ags, large Ags are initially excluded from follicles and can be localized in the interfolloicular and medullary regions, subcapsular, cortical and medullary lymphatic sinuses, and in some cases in

association with local macrophages or DCs. B cells migrate to these regions in a random fashion and can acquire cognate Ags at these sites (83-86). Additionally, B cells can acquire large Ags from DCs that migrate to the LNs and bring internalized Ags from upstream lymphatics to the interfollicular areas and T-B border (87). A non-degradative pathway of Ag recycling observed in DCs promotes retention of intact Ag available for B cell acquisition on these cells (33). Ags bound by complement or pre-existing antibodies can be delivered to the follicle by naïve B cells, where they are deposited near the middle of the follicle on follicular dendritic cells (FDCs), large stromal cells with extensive dendritic processes and high expression of the complement receptors CD21 (CR2) and CD35 (CR1) (refs.83, 85, 88, 89). Ags deposited on FDCs can remain intact for extended periods of time; they can cycle through non-degradative compartments and resurface periodically, where they are available for acquisition by Ag-specific B cells (90, 91).

Although in some cases activated Th cells have been observed to localize to the T-B border as early as 1 day after immunization, the timing of Th cell help availability to activated B cells depends on the time required for DCs to acquire Ag in the periphery, become activated, and migrate to draining LNs or acquire Ag locally within interfollicular and medullary sinuses and activate Ag-specific T cells, whose frequency is usually very low ($1:10^5$-10^6) (refs.92, 93, 94). Moreover, specific MHCII restrictions, various diseases, genetic disorders, age, and immunosuppressive therapies can reduce the number of cognate Th cells or delay their activation (95, 96). For example, HLA class is one of the major genetic determinants of widespread variability in immune responses to a number of vaccines, and this is attributed to variability in efficiency of binding to various antigenic peptides among classes of HLA (96-98) While the timing of Ag stimulation and activation of B and cognate Th cells is likely to differ widely depending on multiple factors, how different dynamics of Ag and T cell help acquisition affect B cell fate remain largely unexplored.

Fate of Ag-primed B cells in the absence of T cell help

Much of the research concerning the fate of Ag-activated B cells that do not acquire T cell help comes from studies of autoimmunity. B cells specific for self-Ag are unlikely to acquire T cell help, as self-reactive T cells are removed from the proinflammatory repertoire more stringently than B cells during development (99). Studies of autoreactive B cells have established a consensus that to maintain tolerance, Ag-activated B cells that do not acquire T cell help must

be removed from the responding repertoire either through death or induction of an unresponsive state termed anergy (100). Tolerance of the primary B cell repertoire is induced at two stages during B cell development. The first is central tolerance in the bone marrow, where developing B cells that bind Ag with high avidity undergo receptor editing or apoptosis. The second stage is peripheral tolerance, when immature B cells egress from the bone marrow and localize to SLOs. Whereas Ag recognition induces activation of mature B cells, it induces death or an unresponsive anergic state in immature B cells, depending on the avidity of Ag binding. High avidity binding induces death, as in the bone marrow, whereas induction of anergy is observed in B cells that undergo constant, relatively low-avidity BCR signaling (101-103). Anergy has been modeled in doubly transgenic mice in which one transgene encodes a constitutively produced soluble form of the small 14kDa protein hen egg lysozyme (HEL), and the second encodes a HEL-specific BCR (Ig-Tg). B cells from these mice exhibit downregulation of IgM, but maintain high IgD expression. When stimulated with Ag and T cell help *in vivo*, they fail to upregulate the co-stimulatory molecule CD86 and generate drastically reduced or undetectable antibody responses (104-106). Maintenance of their unresponsive state requires continual engagement of the BCR, as anergic B cells transferred to non-transgenic mice recover high surface expression of IgM between 4 and 10 days and can generate antibodies in response to Ag and T cell help. However, removal from continual exposure to soluble Ag does not completely reverse anergy, as this response is impaired compared to non-anergic B cells, even if provision of Ag and T cell help is delayed until expression of surface IgM is recovered (105). Downregulation of IgM but not IgD observed in these cells may be due to the selective signaling of IgD in response to multivalent Ags (107).

In addition to reduced responsiveness to Ag, anergic B cells have reduced lifespans compared to naïve mature B cells, and like Ag-activated non-anergic cells they are excluded from follicles and localize near the T cell zones of SLOs. While this relocalization is dependent on CCR7, it is due to decreased expression of CXCR5 relative to naïve and activated non-anergic cells rather than upregulation of CCR7 (refs. 106, 108). Follicular exclusion of anergic B cells correlates with their reduced lifespan; one reason for this could be their increased dependence on BAFF to compensate for upregulated levels of the proapoptotic protein Bim induced by chronic BCR signaling (109, 110). As BAFF is abundantly produced in follicles by FDCs, follicular

exclusion of anergic B cells may impair their survival. Indeed, overexpression of BAFF or deletion of Bim can rescue anergic B cells from death (110, 111).

Anergy does not require exposure to self Ag from the immature stage of development; it can be induced in mature cells, as when mature naïve Ig-Tg B cells are transferred to soluble HEL-expressing mice. As with B cells exposed to Ag throughout development, selective downregulation of IgM is observed within 20 hours of transfer, and transferred cells are functionally unresponsive within 2 days of transfer; immunization and transfer of activated cognate helper T cells at this time elicits severely impaired antibody responses (112). On the other hand, immediate availability of T cell help enables robust GC and antibody responses by non-anergic B cells even in a tolerogenic environment (113). Similar follicular exclusion, localization to the T cell areas, and Bim-dependent disappearance within approximately 3 days are also observed in Ig-Tg mice transferred to soluble HEL-expressing recipients (106, 111, 113).

As demonstrated by many studies, persistent acquisition of self-Ag by B cells that do not acquire T cell help results in anergy or apoptosis. However, in a primary immune response against foreign Ag, T cell help is most likely to be limited during the earliest stages, when large Ags are most restricted from the follicles and less available for ready B cell acquisition. Studies visualizing B cells' acquisition of large Ags trapped in the SCS at the edge of the follicle during these very early stages of the response found that these encounters were brief (85, 114, 115). Technical limitations made it impossible to determine the fate of those B cells, but these studies suggest that early B cell encounters with large foreign Ags may be brief as opposed to continuous, as in autoimmunity studies, raising the question of whether B cell fate might be different after transient Ag acquistion. One study addressed this question using B cells transiently primed with Ag and cultured *ex vivo* and found that a single round of Ag acquisition stimulated transient NFκB signaling and increased sensitivity to CD40L, potentially priming B cells to receive T cell help, but unlike continuous Ag stimulation, was insufficient to initiate cell cycling and impaired B cell survival (116). In Chapter 2, the fate of B cells is addressed following transient Ag acquisition *in vivo*, whether they can be recruited into the T-dependent humoral response and what becomes of them in the absence of T cell help.

Recruitment of B cells into GCs

The ideal vaccine should lead to generation of broadly neutralizing antibodies against infection. In addition, a diverse repertoire of effector cells is likely important for protection against mutating pathogens or re-infection by a pathogen strain with related but distinct epitopes. However, typical immunizations generate relatively pauciclonal long-lived PC responses. Due to the importance of GCs in generating long-lived high affinity effector cells, activated B cells' recruitment into GCs is an area of active study. Relative affinity for Ag plays a role, as low-affinity B cells inherently capable of GC participation are recruited much less efficiently when competing with higher affinity B cells (55). This is likely at least partially due to higher-affinity B cells' ability to acquire more Ag and compete more successfully for T cell help (54). A secondary selection mechanism could be stronger upregulation of BAFFR by higher-affinity B cells promoting their survival after initial activation, enabling preferential GC recruitment (117, 118). Roughly tens to hundreds of clones are recruited per GC by 6 days after immunization with large protein Ag (119). Beyond this initial population, the clonal diversity of ongoing GCs can be increased by recruitment of recirculating Ag-specific B cells that enter Ag-draining SLOs (120). However, the factors limiting recruitment of newly entering B cells into the ongoing GC response are not known.

Several factors may limit recruitment of newly activated B cells into GCs. The population of follicular T cells is dynamic, undergoing changes in the ratio of Tfh to Tfr cells and changing cytokine profile over the course of the GC response. GC-supporting Tfh cells first undergo rapid expansion and later contract; whether limiting numbers of these cells can reduce GC entry of newly activated B cell clones is not known. This contracted population of Tfh cells also makes less IL-21 during later stages of the GC response; this cytokine is necessary for GC maintenance and promotes GC B cell proliferation (73, 74). Finally, the frequency of Tfr cells, which negatively regulate GCs, is increased during the later stages of GC responses (79, 121). In addition to the changes in Tfh and Tfr cells, access of naïve B cells to Ag deposited in immune complexes on FDCs in the GC declines over time; this may be mediated by increased competition with high-affinity GC B cells or steric hindrance by higher affinity antibodies from PCs differentiating from GCs preventing Ag acquistion by lower affinity new recruits (91, 122). In Chapter 3, we address which of the factors described above may affect the recruitment of new B cell clones entry into ongoing immunization-induced GC responses.

Memory B cell differentiation

Memory responses are critical for long-term protection against previously encountered pathogens and are characteristically differentiated from primary B cell responses by their increased speed, greater magnitude, higher affinity, and increased frequency of isotype-switched antibodies. Because affinity maturation and isotype switching mostly occur in GCs, it was historically believed that memory B cells were predominantly GC-derived, and memory cells were phenotypically defined as isotype switched B cells that were negative for GC or PC markers. However, more recent studies demonstrated that a population of unswitched memory B cells exists, and that both switched and unswitched memory B cells can be generated independently of GCs (51, 123, 124). The definition of memory B cells has consequently expanded, and at its most broad represents Ag-experienced cells that persist in its absence and produce a more robust response upon Ag reencounter than that of naïve B cells (125, 126).

This definition encompasses functionally distinct subsets of memory B cells that have been identified phenotypically in mice either by expression of an isotype-switched or unswitched BCR or by expression of combinations of the B7 family members CD80 and PD-L2 (refs. 29, 51, 123, 124, 127). Studies identifying these functional subsets found that IgM or double negative (DN, $CD80^{-}PD\text{-}L2^{-}$) memory cells are more "naïve-like" in that they are more predisposed to participate in secondary GCs prior to differentiating into PCs, whereas isotype switched, single positive (SP, $CD80^{-}PD\text{-}L2^{+}$), or double positive (DP, $CD80^{+}PD\text{-}L2^{+}$) memory B cells are more "effector-like," in that they differentiate more rapidly into PCs upon reencountering Ag and T cell help (123, 124, 127). This could be due to decreased expression in class-switched memory B cells of the transcription factor BTB and CNC homologue 2 (Bach2), which suppresses PC differentiation (128). Of note, isotype-switched and unswitched memory B cells were identified in all three B7-defined subpopulations, but the DN population consists of predominantly IgM^{+} B cells, whereas the SP and DP populations are comprised of progressively more class-switched cells, with DP more effector-like than SP (127). Analyses of memory B cell differentiation kinetics suggest that naïve-like memory B cells are generated predominantly prior to formation of GCs, whereas the majority of memory B cells that differentiate from GCs belong to the effector-like subsets (52, 124).

The factors influencing differentiation and maintenance of memory cells are not well understood. A "master" transcription factor that promotes memory B cell differentiation, like Blimp1 for PCs or Bcl6 for GC B cells, has not been identified, and it is has been suggested that

memory B cell differentiation may be a default pathway for the surviving population of responding B cells that are not recruited into the PC response (28). In support of this hypothesis, it was demonstrated that increasing overall survival of B cells through overexpression of the prosurvival factor Bcl2 expands the memory cell population without disrupting selection of high affinity PCs (129). However, there is also evidence for an instructional model, in which memory B cells are actively selected to differentiate based on various inputs such as extrinsic signals from T cells or affinity-based signals through the BCR. Production of IL-9 by Tfh cells and signaling through IL-9R on GC B cells can promote memory B cell differentiation from GCs (130). Memory B cells tend to be lower affinity than PCs, even those that differentiate from GCs. Most GC-derived memory B cells are generated in the earlier stages of the GC response, and consequently have lower levels of somatic hypermutation (52, 124, 131). A potential mechanism behind selection of lower affinity GC B cells for differentiation into memory cells is that T cell help, which is thought to be preferentially acquired by high-affinity GC B cells, decreases expression of Bach2, which promotes memory B cell differentiation during the early stages of the GC response (131-133). However, few memory B cells are produced from later GCs, regardless of Bach2 expression, indicating that other factors play necessary roles in memory B cell differentiation (131).

While the influence of Tfh cell-derived signals on memory B cell differentiation are actively being studied, how dynamics of Ag acquisition alter memory B cell differentiation and generation of the different memory B cell subsets is unknown. In Chapter 4, we address whether transient acquisition of Ag by B cells is sufficient for development of memory cells, which subsets of memory cells can be formed, and how long they persist compared to those generated in response to recurrent acquisition of Ag.

GC B cell selection

GCs have been observed as histologically distinct inducible structures for over a century and are characteristically polarized into the dark zone (DZ), named for the densely packed proliferating GC B cells it houses, and the light zone (LZ), so named for its decreased density of nuclei relative to the DZ; it houses FDCs and the majority of follicular T cells in addition to GC B cells. The two zones are thought to be functionally distinct, with the DZ being the predominant site of GC B cell proliferation and hypermutation and the LZ where GC B cells compete for Ag

and T cell help (57). LZ and DZ B cells cycle between the two zones and can be differentiated phenotypically by increased expression of co-stimulatory molecules CD83 and CD86 by LZ B cells, and increased expression of CXCR4 on DZ B cells (133). GC polarization is dependent on upregulation of CXCR4 by DZ B cells, which drives their migration towards CXCL12, a chemokine produced by a network of reticular cells in the DZ. However, GC B cells lacking CXCR4 still cyclically upregulate CD83 and CD86 and undergo affinity maturation, although less efficiently than GC B cells that cycle between the zones normally (134, 135).

Efficient selection of GC B cells with increased affinity for Ag is critical for driving affinity maturation and generation of high affinity PCs, and has consequently been an active area of investigation. There are two non-mutually exclusive models of GC B cell selection. The first model suggests that stronger BCR signaling by cells with higher affinity BCRs promotes their survival and selection, whereas the second model proposes that survival signals are derived from Tfh cells and that GC B cells with higher affinity BCRs are selected due to their increased acquisition of Ag for presentation to Tfh cells.

Although GC B cells have increased phosphatase activity that suppresses BCR signaling during most phases of the cell cycle, phosphatase delocalization from the BCR and phosphorylation of Syk were observed in response to BCR ligation with soluble anti-IgM in GC B cells in the G2 phase of the cell cycle, indicating that BCR signaling may be active during G2 and have a role in GC B cell selection (136). In contrast to acquisition of soluble Ag, an *ex vivo* imaging study of splenic GC B cells acquiring highly multivalent Ag from immobilized planar lipid bilayers demonstrated robust phophorylation of Syk and signalosome components, as well as calcium signaling and activation of the Ras/MAPK cascade. Notably, nuclear translocation of NF-κB was not induced by Ag binding, but was by CD40 signaling (137). GC B cells are thought to primarily acquire immobilized Ag from FDCs, and these results indicate that BCR signaling in GC B cells may be at least partially active depending on the context of Ag acquisition. Additional support for the BCR signaling model of GC B cell selection comes from a study demonstrating *in vivo* BCR signaling in a subset of LZ B cells as indicated by a fluorescent reporter of Nur77, a gene rapidly upregulated in response to BCR signaling through Syk and partially dependent on PKC, calcium signaling, MAPK, and PI3K activation in naïve cells, although the signaling pathway utilized by the GC B cells in which Nur77 was upregulated was not determined (138, 139). Finally, a study of early stages of PC differentiation

demonstrated that although a similar decrease in overall size of the GC was induced by blocking GC B cell access to Ag and depletion of Tfh cells, loss of Ag availability resulted in a stronger defect in early GC B cell differentiation into PCs, indicating that BCR signaling may be particularly important for initiating PC differentiation in GC B cells (140).

Many studies have illustrated the critical role for Tfh cells in GC B cell survival and selection. Some of the key molecules identified as contributing to T cell help in the GC and promoting survival, class-switching, and effector differentiation include CD40, ICOS, and the cytokines IL-21, IFNγ, and IL-4. One of the most important signals provided by Tfh cells is CD40; blocking it alone is sufficient to halt ongoing GCs, and it promotes PC differentiation by downregulating Bach2 as well as by increasing Blimp1 when combined with IL-21 (refs. 72, 131, 141). ICOS/ICOSL interactions are critical for the intimate contacts between GC B cells and Tfh cells and enhance CD40 signaling (70, 71). Finally, Tfh production of the cytokines IL-4 and IFN-γ are important for promoting class switching to IgG1 and IgG2a, respectively (73, 142-144).

Because both Ag and Tfh cells are critical for maintenance of GCs, their individual contributions to GC B cell selection have been difficult to determine. Several recent studies have provided support for the T cell help-based model of selection. Delivery of Ag to GC B cells in a manner that bypassed the BCR but enabled peptide presentation in MHCII promoted their proliferation and differentiation into PCs (133). Additional studies using this system demonstrated that Ag delivered in this fashion increased the speed of the cell cycle in GC B cells and that the extent of proliferation and hypermutation was proportional to the amount of Ag delivered (145, 146). Additional support for the T cell help-based model of selection comes from the finding that turnover of MHCII in GC B cells is required for their efficient selection (147). Taken together, these studies indicate that provision of additional T cell help to GC B cells can promote their proliferation and PC differentiation. However, whether integration with signals from the BCR is necessary for this enhancement, or whether increased BCR signaling can promote GC B cell survival or selection alone or in combination with T cell help have not been addressed. In Chapter 5, we sought to address the roles of acquisition of Ag and T cell help individually and in combination in promoting GC B cell survival, selection, and effector differentiation.

Scope of the Thesis

Many factors regulating B cell recruitment into the T-dependent humoral immune response and influencing their effector differentiation have been determined. However how the dynamics of their acquisition of Ag and T cell help affect these processes is poorly understood. My research has focused on how these dynamics affect B cell recruitment into various stages of the T dependent humoral immune response and how their effector differentiation is altered by varied timing of acquisition of Ag and T cell help. In Chapter 2, the fate of B cells following transient Ag acquisition and their ability to participate in the T-dependent humoral response is addressed when T cell help is immediately available or when it is delayed. In Chapter 3, the ability of Ag acquisition to enable naïve B cells' recruitment into GCs is addressed during different phases of the GC response. In Chapter 4, differentiation of different subsets of memory B cells is addressed following transient or recurrent Ag acquisition. Finally in Chapter 5, the roles of Ag acquisition and T cell help in GC B cell selection are addressed in a preliminary fashion; experiments evaluating their individual roles are ongoing.

Chapter 2. Transiently antigen-primed B cells return to a naive-like state in the absence of T-cell help

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Abstract

The perspective that naïve B cell recognition of antigen (Ag) in the absence of T cell help causes cell death or anergy is supported by *in vivo* studies of B cells that are continuously exposed to self-Ags. However, intravital imaging suggests that early B cell recognition of large foreign Ags may be transient. Whether B cells are tolerized or can be recruited into humoral immune responses following such encounters is not clear. Here we show that in the presence of T cell help, single transient Ag acquisition is sufficient to recruit B cells into the germinal center and induce memory and plasma cell responses. In the absence of T cell help, transiently Ag-primed B cells do not undergo apoptosis *in vivo*; they return to quiescence and are recruited efficiently into humoral responses upon reacquisition of Ag and T cell help.

Introduction

In accord with the two-signal model of lymphocyte activation proposed by Bretscher and Cohn, B cells must receive a primary activating signal through Ag-dependent crosslinking of B cell receptors (BCRs) and a second signal from activated cognate helper T (Th) cells to get recruited into long-term, high-affinity humoral immune response (30, 31, 148, 149). In secondary lymphoid organs, after Ag binding triggers BCR signaling, activated B cells internalize the Ag by receptor-mediated endocytosis and then undergo a series of phenotypic and migrational changes (11). Within 6 hours, activated B cells upregulate expression of the costimulatory molecule CD86 (ref. 44) and present proteolyzed antigenic peptides in complex with MHCII for recognition by cognate Th cells (43). They also upregulate CCR7, a G-protein coupled receptor specific for the T zone chemokines CCL19 and CCL21 and migrate from B cell

follicles to the B-T zone border and interfollicular area where they can interact with cognate Th cells (38, 45, 46). After acquisition of T cell help, B cells undergo proliferation and differentiate into short-lived plasma cells (PCs) and germinal center (GC) B cells. Although B cells with a wide range of BCR affinities to Ag are recruited into T-dependent B cell response *in vivo*, the clones with lower Ag binding strengths generate reduced short-lived PC responses, due to limited expansion of plasmablasts, and populate GCs less when faced with interclonal competition for T cell help (53-55, 150). Within GCs, B cells then undergo somatic hypermutation of the BCR and compete for Ag deposited on follicular dendritic cells (FDC) and help from follicular helper T cells in a process that drives GC B cells' affinity maturation and formation of high affinity class-switched memory cells and long-lived PCs (57). Although many studies have focused on the cellular, molecular, and chemotactic mechanisms regulating B cell response initiation and GCs, how temporal dynamics of Ag and T cell help acquisition by B cells affect engagement with T cell-dependent humoral responses *in vivo* is unclear.

The dynamics of B cells' exposure to foreign Ag *in vivo* depend on multiple factors, including the Ag's physical properties, route of entry, and formation of immune complexes. Small Ags (e.g. toxins, proteolyzed pathogen fragments) quickly permeate B cell follicles, whereas initially, large Ags are often restricted to the subcapsular and medullary sinuses and interfollicular areas of the lymph nodes (151). By 2-photon imaging it has been shown that, during initiation of the B cell response, naïve Ag-specific B cells can transiently approach these regions (for a few minutes to a few tens of minutes), acquire the large Ags, and then return to B cell follicles (85, 114, 115). However, due to technical limitations, the precise history of Ag acquisition by these cells and their fate has not been possible to study.

A previous *ex vivo* study of B cell signalling and transcriptional regulation suggests that a single round of BCR signalling may be sufficient to prime B cells for acquisition of T cell help. However, it also suggests that survival of transiently Ag-primed B cells in the absence of T cell help is compromised (116). This observation is consistent with Polly Matzinger's hypothesis that to maintain tolerance, B cells that acquire Ag but not T cell help must die (100). Supporting this proposal, multiple *in vivo* studies demonstrated that B cells that continuously acquire self-Ag undergo apoptosis or anergy (152, 153). However, the fate *in vivo* of B cells transiently exposed to Ag is unclear, both with respect to induction of tolerance and recruitment into T cell-dependent humoral immune responses. Here we show that transient Ag acquisition enables B cell

participation in GC, memory B cell, and PC responses when T cell help is available and allows B cells to return to a naïve-like state when it is not, rather than undergo anergy or apoptosis.

Materials and Methods

Mice. B6 (C57BL/6) mice were purchased from Charles River, NCI, or the Jackson Laboratory. B6-CD45.1 (Ptprc^{a} Pepc^{b}/BoyJ) and TCRα$^{-/-}$ (*Tcra*^{tm1Mom}) mice were purchased from the Jackson Laboratory. BCR transgenic (Ig-Tg) Hy10 (ref. 154) and MD4 mice (104) and TCR transgenic OTII mice (155) (all C57BL/6 background) were generously provided by Jason Cyster. Ig-Tg MD4 and Hy10 mice were crossed with B6-CD45.1 mice and maintained on this background. Hy10 B cells are capable of Ig class switching and were used for experiments characterizing the germinal center response (**Fig. 2.1** and and **Fig. 2.3**). MD4 Ig-Tg mice have a more highly penetrant transgene, allowing for higher recovery of Ig-Tg B cells (~95% of total B cells), and were used in all other experiments. Donor and recipient mice were 6–12 weeks of age. All mice were maintained in a specific pathogen free environment and protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Ag preparation. Duck eggs were locally purchased and duck egg lysozyme (DEL) was purified as previously described (154). Hen egg lysozyme (HEL) and ovalbumin (OVA) were purchased from Sigma. DEL or HEL was conjugated to OVA via glutaraldehyde cross-linking as previously described (154). For conjugation of DEL-OVA to an I-E alpha chain peptide containing the epitope Eα 52-68 (Eα peptide), Eα peptide with four amino acids from the native sequence flanking each end and a cysteine replacing the N-terminal phenylalanine to allow for maleimide targeting was purchased from GenScript (CAKF**ASFEAQGALANIAVDKA**NLDV). This peptide was crosslinked to DEL-OVA at 10 fold molar excess using bismaleimidoethane (Pierce) according to the manufacturer's directions.

For generation of multivalent DEL (mDEL), DEL was combined with biotin-NHS (Fisher) at a 1:2 molar ratio and conjugated and purified according to the manufacturer's directions. Biotinylated DEL was incubated 30' on ice with purified streptavidin (Sigma) at a 10:1 molar ratio and the conjugate was purified with 40 kDa desalting columns (Bio-Rad) according to the manufacturer's directions.

For generation of DEL-coated microspheres (sphDEL), 0.11 μm streptavidin coated polystyrene microspheres (Bangs Laboratories) were dialyzed into PBS and combined with a saturating amount of DEL-bio as described previously (156).

Adoptive transfer and immunization. Spleens were harvested from male donor OTII mice and pressed through 70 μm nylon cell strainers in DMEM supplemented with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 2% FBS, 10 mM HEPES, 50 IU/mL of penicillin, and 50 μg/mL of streptomycin (HyClone), (DMEM 2%). Splenocytes were centrifuged for 7 minutes at 380 x *g*, 4 °C and resuspended in 0.14 M NH_4Cl in 0.017 M Tris buffer, pH 7.2 for erythrocyte lysis, washed twice with DMEM 2%, and counted using a Cellometer Auto X4 (Nexcelom). The fraction of $CD19^-$ $CD8^-$ $CD4^+$ $V\beta5^+$ (OTII) splenocytes was determined by flow cytometry, and $5x10^5$ OTII cells were transferred i.v. to male B6 recipient mice, which had been recently purchased or bred in-house.

Ig-Tg and wild-type B cells were enriched from donor mice by negative selection as previously described (134). For labeling with carboxyfluorescein succinimidyl ester (CFSE), purified Ig-Tg or wild-type B cells were washed and resuspended in DMEM supplemented as above, but with 1% FBS at fewer than 10^7 cells per mL. CFSE was added to the cells at a final concentration of 1 μM, and cells were incubated for 20 min at 37 °C. 2 mL of FBS was layered under the cells, which were centrifuged for 7 min at 380 x *g*, 4 °C and resuspended in DMEM 2%.

For transient exposure to Ag, purified Ig-Tg B cells were incubated with HEL, HEL-OVA, DEL-OVA, DEL-OVA-Eα, mDEL, or sphDEL *ex vivo* for 5 minutes at 37 °C, washed four times with room temperature DMEM 2%, mixed with CFSE-labeled naïve control cells where indicated, and transferred i.v. to recipient mice or cultured in DMEM supplemented as above with 10% FBS and 50 μM β-mercaptoethanol.

Where indicated, recipient mice were immunized i.p. with 50 μg OVA or DEL-OVA in Ribi (Sigma), or s.c. with 50 μg OVA, HEL-OVA, or DEL-OVA emulsified in complete Freund's adjuvant (Sigma), prepared according to the manufacturer's directions.

For transfer of Ig-Tg and control cells from primary (1°) to secondary (2°) recipient mice, single cell suspensions of splenocytes from 1° recipients were made and treated for erythrocyte lysis as above, resuspended in DMEM 2%, counted, and transferred i.v. to 2° recipients. At least

2 million splenocytes from 1° recipients were reserved for flow cytometry staining to determine the ratio of Ig-Tg to control cells. For experiments in which unproliferated Ag-pulsed and naïve Ig-Tg B cells were sorted from 1° recipients prior to transfer into 2° recipients (**Fig. 2.9e**, **g**), splenocytes from 1° recipients were either stained directly for sorting or depleted of non-B cells by negative selection as above, then stained for sorting with fluorochrome-conjugated CD19, CD4, CD8, CD45.1, and CD45.2 diluted in PBS supplemented with 0.5% FBS, 2mM EDTA, and 10 mM HEPES (sorting buffer) for 20 min on ice. Cells were washed twice and resuspended in sorting buffer. $CD19^+$ $CD4^-$ $CD8^-$ $CD45.1^+$ $CD45.2^-$ $CFSE^{high}$ Ig-Tg B cells and $CD19^+$ $CD4^-$ $CD8^-$ $CD45.1^-$ $CD45.2^+$ $CFSE^{high}$ control B cells were sorted on a FACSAria and resuspended in DMEM 2%. Within each experiment, the same number of inactivated or naïve B cells ($4–7x10^3$) were transferred i.v. to 2° recipients, which were re-immunized with 50 μg DEL-OVA in Ribi, i.p.

Flow cytometery. Single-cell suspensions from spleens or lymph nodes were incubated with biotinylated antibodies (**Table 1**) for 20 minutes on ice, washed twice with 200 μl PBS supplemented with 2% FBS, 1 mM EDTA, and 0.1% NaN_3 (FACS buffer), incubated with fluorophore-conjugated antibodies and streptavidin (**Table 1**) for 20 minutes on ice, washed twice more with 200 μl FACS buffer, and resuspended in FACS buffer for acquisition. For intracellular staining, surface-stained cells were fixed and permeabilized for 20 minutes on ice with BD Cytofix/Cytoperm buffer, washed twice with 200 μl BD Perm/Wash buffer, incubated with Alexa 647-conjugated HEL for 20 minutes on ice, followed by two washes with 200 μl Perm/Wash buffer, and resuspended in FACS buffer for acquisition. Data were acquired on a FACSCanto or LSRFortessa and analyzed using FlowJo (TreeStar).

ELISAs. Serum was collected from recipient mice serially bled through the saphenous or tail vein or terminally bled through the portal vein by incubating blood for 20’ at room temperature in polypropylene microcentrifuge tubes followed by centrifugation for 10’ at 2000 x *g*, 4 °C. Supernatant was collected and stored at -20 °C until assayed. 96-well polyvinylchloride plates were coated overnight at room temperature in a humid chamber with 100 μl 10mg/mL HEL in 22 mM sodium carbonate, 34 mM sodium bicarbonate buffer, 0.2% $MgCl_2$, pH 9.8. The wells were then blocked for 4 h with 100 μL 10% FBS, 0.04% NaN_3 in PBS at room temperature in a humid chamber and washed once with 0.05% Tween 20 in PBS (PBST). Five-fold serial

dilutions of sera in duplicate were added to the wells with three-fold dilutions of appropriate standards (pooled sera from MD4 mice and purified HyHEL10 IgG_1 for IgM^a and $IgG_1{}^a$, respectively) and the plates were incubated 2 h at room temperature in a humid chamber. Wells were washed four times with 200 μL PBST, and 100 μL biotinylated IgM^a and $IgG_1{}^a$ antibodies diluted 1:4000 and 1:1000, respectively in 1% BSA, 0.02% $MgCl_2$, 0.02% NaN_3 Tris buffer, pH 8 were used to detect bound serum antibody. Plates were incubated overnight at room temperature in a humid chamber and washed four times with 200 μL PBST. Alkaline phosphatase-conjugated streptavidin was diluted 1:4000 in Tris buffer, and 100 μL was added to the wells. Plates were incubated 2 h and washed four times with 200 μL PBST. 100 μL 1mg/mL p-nitrophenyl phosphate in carbonate buffer was added to the wells, and absorbance at 405nm was measured with a Synergy HT microplate reader (BioTek Instruments). The concentrations of anti-HEL antibodies were calculated from standard curves generated from a stock of pooled serum from naïve MD4 mice for IgM^a and purified HyHEL10 antibody for $IgG_1{}^a$. Significant differences were determined by unpaired, two-tailed Student's t test in Prism (GraphPad).

Immunofluorescence. Spleens and ILNs were harvested from recipient mice, fixed for 1 h in 1% paraformaldehyde in PBS on ice, washed with PBS, blocked overnight in 30% sucrose, 0.1% NaN_3 in PBS, embedded in Tissue-Tek optimum cutting temperature compound, snap-frozen in dry ice and ethanol, and stored at -70 °C. Thirty micron cryostat sections were cut from the tissue blocks, affixed to Superfrost Plus microscope slides (Fisher), and stained with IgD, CD45.1, and Bcl6 or CD3, CD19, and CD45.1 as previously described (148). Slides were analyzed at room temperature using a Leica SP5 with argon and helium-neon lasers, 2-channel Leica SP spectral fluorescent PMT detector, and a 20x oil-immersion objective with a numerical aperture of 0.7. Images were processed using Imaris (Bitplane) and analyzed using ImageJ (NIH) and Matlab (Mathworks). For B cell localization analysis, the average normalized distance of Ig-Tg cells to the T/B border in a follicle was defined as the average distance to the T/B border of the Ig-Tg B cells in the follicle divided by the average distance to the T/B border of points randomly distributed within the follicle, such that 0 corresponds to cell distribution along the T/B border, and 1 to random distribution within the follicle.

Statistics. Statistical tests were chosen in consultation with the University of Michigan Center for Statistical Consultation and Research and performed as indicated using Prism 6 (GraphPad). Differences between groups not annotated by an asterisk did not reach statistical

significance. Power calculations to determine sample size for experiments making statistical comparisons between groups were performed after initial experiments enabled estimation of effect size. No blinding or randomization was performed for animal experiments, and no animals or samples were excluded from analysis.

Results

Ag primed B cells are recruited into humoral responses

To determine the fate of B cells after a single transient acquisition of Ag *in vivo* we utilized the following approach. BCR transgenic (Ig-Tg) HyHEL10 B cells specific for hen egg lysozyme (HEL) (154) were pulsed *ex vivo* for 5 minutes with HEL fused to ovalbumin (HEL-OVA), unbound Ag was washed off, and the cells transferred into recipient mice, which had been preinjected with transgenic OTII Th cells specific to peptide $ova_{323-339}$ in I-A^b (ref. 155) and preimmunized with OVA in complete Freund's adjuvant (CFA) (**Fig. 2.1a**). While HEL-OVA-primed B cells could not reacquire cognate HEL Ag *in vivo*, they could digest pre-acquired OVA, present OVA-derived peptides, and make cognate interactions with activated OVA-specific Th cells. In inguinal lymph nodes (ILN) of OVA-immunized recipient mice, Ag-primed B cells underwent proliferation and were transiently recruited into GCs (**Fig.2.1b–e**, **Fig.2.2**). They also differentiated into memory B cells ($CFSE^{low}$ $GL7^{low}$ $CD38^{high}$, both IgD^{pos} and class-switched) and PCs, and generated a modest class-switched antibody (Ab) response, predominantly of the IgG_1 isotype (**Fig. 2.1f–i**, **Fig.2.2**). Recruitment of Ig-Tg cells into the B cell response was dependent on cognate interactions with activated helper T cells, since Ig-Tg B cells pulsed with HEL did not form GCs or PCs in OVA immunized recipient mice (**Fig. 2.1e**, **g**, **day 6**, hashed bars), similarly to HEL-OVA pulsed Ig-Tg cells in unimmunized control mice (**Fig. 2.1a**, **e**, **g**). To verify these results were not an artifact of high affinity Ag, Ig-Tg B cells were primed with duck egg lysozyme (DEL) Ag conjugated to OVA, which has a more physiologic, 10^3-fold lower affinity to Ig-Tg BCRs compared to HEL (157). Comparable participation of Ig-Tg cells in the B cell response was observed following priming with HEL-OVA and DEL-OVA (**Fig. 2.1j–l**). Together, these data suggest that a single transient Ag acquisition may be sufficient to enable B cells' participation in the T-dependent B cell response *in vivo*.

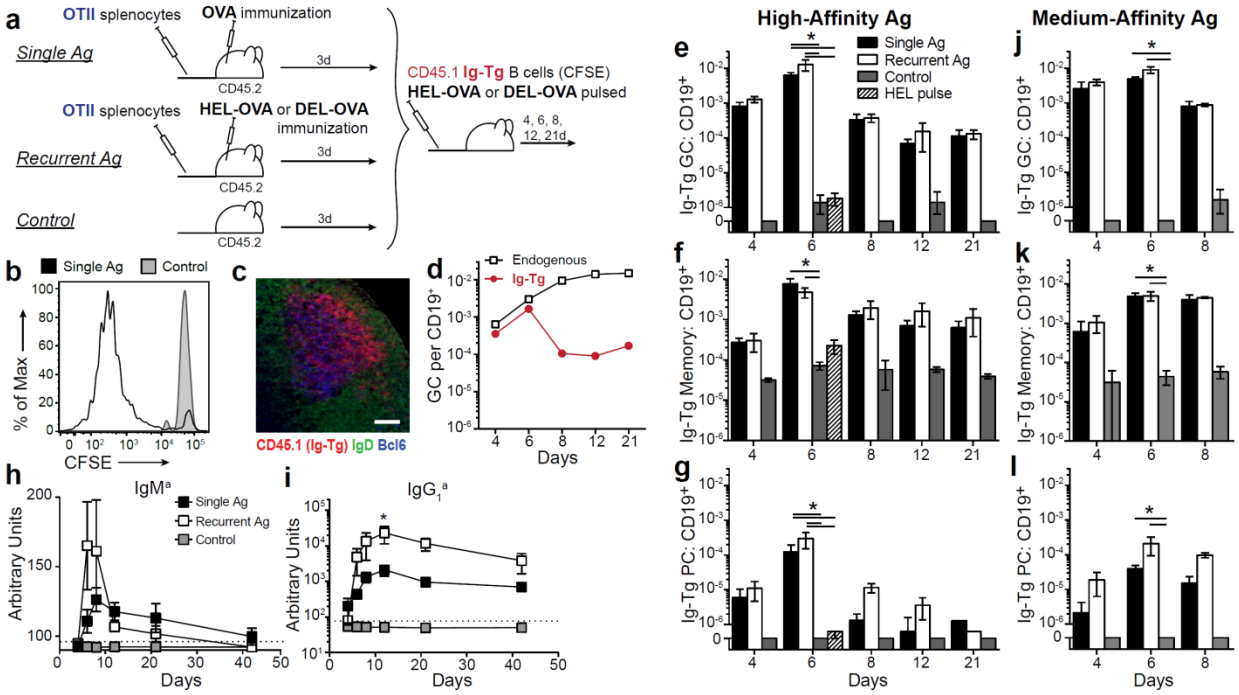

Figure 2.1. A single exposure to Ag enables B cell participation in the GC, memory B cell, and PC responses *in vivo*. a, Experimental outline.**.** **a**, Experimental outline. CFSE-labeled HyHEL10 Ig-Tg B cells were pulsed *ex vivo* for 5 min with 50 μg/mL HEL-OVA or DEL-OVA, washed and transferred into recipient mice preinjected with OTII Th cells and subcutaneously (s.c) preimmunized with OVA, HEL-OVA, or DEL-OVA in CFA or into unimmunized control mice. **b–d**, Recruitment of HEL-OVA pulsed Ig-Tg B cells into the B cell response in draining inguinal LNs (ILNs) of mice immunized with OVA. **b**, Proliferation of Ag-pulsed Ig-Tg cells 4 days post transfer (d.p.t) in OVA-immunized (Single Ag) or unimmunized (Control) recipient mice. **c**, Confocal micrograph of $IgD^{low}Bcl6^{+}$ GC at 6 d.p.t. Scale bar = 70 μm. **d**, Kinetics of endogenous (white boxes) and Ag-pulsed Ig-Tg (red circles) B cells' participation in GC response. Representative of n=3 (**b**, **d**) or n=2 (**c**) independent experiments. **e–l**, Kinetics of HEL-OVA (high affinity Ag, **e–i**), DEL-OVA (medium affinity Ag, **j–l**), or HEL (1 μg/mL, hashed bar, **e–g**, d 6 only) pulsed Ig-Tg B cells' participation in the GC, memory, PC, and antibody response in mice immunized with OVA (black and hashed symbols), HEL-OVA (**e–i**, white symbols), DEL-OVA (**j-l**, white symbols) in CFA or in unimmunized mice (grey symbols). See also **Fig. 2.2**. Data are from ILNs, n=3 independent experiments, 3 mice per condition, except for d 6, which is from n=7 independent experiments, 9 mice per condition (**e–g**, **j–l**, HEL-OVA and DEL-OVA pulsed) or 2 independent experiments, 6 mice per condition (**e–g**, HEL pulsed). Data shown as mean ± SEM. *, P<0.05 (Kruskal-Wallis test with Dunn's post-test). **h**, **i**, Serum αHEL IgM^{a} (**h**) and the predominant class-switched IgG_1^{a} (**i**) antibody titers. Dotted line represents ELISA limit of detection. Data are from n=4 independent experiments with 4–5 mice per timepoint. Data shown as mean ± SEM. *p<0.05 (unpaired two-tailed Student's t test between Single and Recurrent Ag).

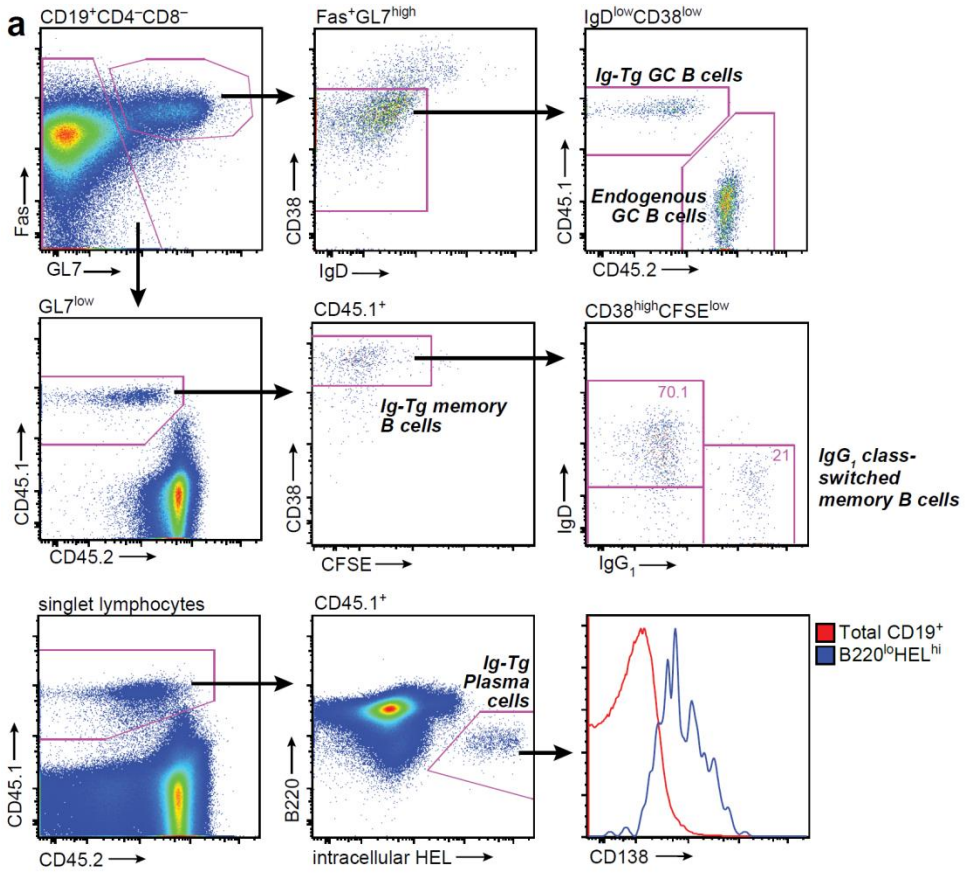

Figure 2.2. Gating strategies for Ig-Tg GC, memory B cells, and PCs. a, Ig-Tg HyHEL10 GC (upper panels), memory B cells and IgG_1 class-switched memory B cells (middle panels), and PCs (lower panels.

Interestingly, initial recruitment of Ag-pulsed Ig-Tg cells into the GC and memory responses in HEL-OVA and DEL-OVA immunized mice, in which Ig-Tg cells could reacquire cognate Ag *in vivo*, was not significantly different from OVA immunized mice (**Fig. 2.1e**, **f**, **j**, **k**). However, we observed a trend for an increased Ig-Tg PC response in HEL-OVA and DEL-OVA immunized recipient mice (**Fig. 2.1g**, **l**, **day 8**), which was reflected in elevated IgG_1^a antibody titers (**Fig. 2.1i**).

Ig-Tg B cells were recruited into the T-dependent B cell response after pulsing with DEL-OVA concentrations ranging from 0.005 to 50 μg/mL (**Fig. 2.3a–h**). Of interest, we found that Ig-Tg B cells formed GC, memory, and PCs even at Ag doses suboptimal for B cell activation (based on their failure to induce CD69 and CD86 upregulation or surface IgM downregulation, **Fig. 2.3a–d**). However, significantly decreased accumulation of PCs was

observed at DEL-OVA concentrations below 0.05 μg/mL, and GC and memory cells at 0.005 μg/mL, consistent with previous studies suggesting greater dependence of PC output on responding B cells' affinity for Ag (**Fig. 2.3e–h**) (53, 54, 150).

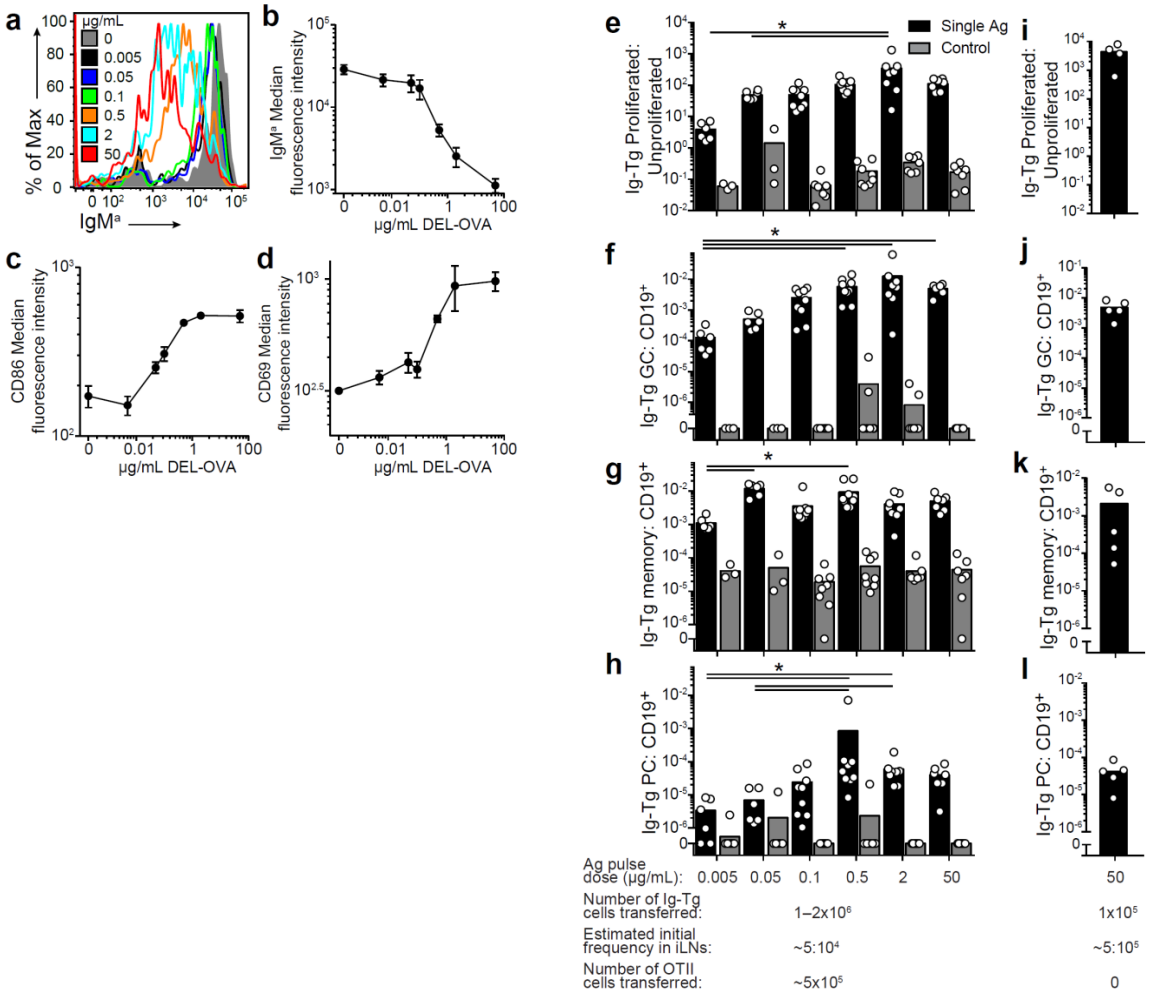

Figure 2.3. A broad range of Ag doses enables recruitment of Ag-primed B cells into T-dependent response *in vivo*. a–d, Ig-Tg B cells' surface IgM^a (BCR) (**a**, **b**), CD86 (**c**), and CD69 (**d**) expression 6h following *ex vivo* pulsing with the indicated doses of DEL-OVA and transfer into naïve recipient mice. Spleens analyzed. Data are representative of (**a**) or from 3 independent experiments, shown as mean ± SEM (**b–d**). **e–l**, Proliferation and formation of GC, memory, and PCs by the indicated number of Ig-Tg B cells pulsed *ex vivo* with the indicated doses of DEL-OVA and transferred into OVA-immunized mice (black bars) or into control unimmunized mice (grey bars) with (**e–h**) or without (**i–l**) OTII Th cells. ILNs analyzed at 6 d.p.t. Each dot represents one mouse and bars correspond to mean values. n=2-5 independent experiments. *p<0.05 (Kruskal-Wallis test with Dunn's post-test).

Moreover, B cell were recruited into the T cell-dependent response when the estimated number of Ag-pulsed Ig-Tg B cells that entered draining LNs was within the range of reported frequencies (10^{-5}–10^{-4}) of endogenous B cells specific to a particular Ag (123, 158) and could be supported by the endogenous OVA-specific Th repertoire (**Fig. 2.3i–l**). Based on these data, we conclude that when T cell help is not limiting, single transient acquisition of Ag over a wide range of doses may be sufficient for the initial recruitment of rare Ag-specific B cells into the T-dependent response.

Ag-primed B cells survive in the absence of T cell help

To study the fate of Ag-primed B cells in the absence of T cell help, we utilized Ig-Tg MD4 B cells, which constitute about 95% of total B cells in MD4 mice, allowing for more straightforward enumeration following adoptive transfer. MD4 B cells express a Tg BCR similar to that of HyHEL10 B cells and similarly bind DEL-OVA (**Fig. 2.3b**, **Fig. 2.4c**), but cannot undergo class-switching (104). To address the fate of Ag-primed B cells in the absence of immediate T cell help *ex vivo* and *in vivo*, Ag-pulsed Ig-Tg B cells were either co-cultured with control naïve B cells or co-transferred into unimmunized recipient mice (**Fig. 2.5a**). Ig-Tg MD4 B cells were pulsed with a high dose of DEL-OVA (50 μg/mL, **Fig. 2a–d**) or DEL-OVA conjugated to E-alpha peptide (Eα) which is recognized in complex with MHCII by the Y-ae antibody and can be used to monitor Ag-derived peptide presentation (159). While previous work and our studies indicate that Ag-primed B cells undergo rapid apoptosis when cultured *ex vivo* (**Fig. 2.4b**) (116), we observed no substantial decrease in the numbers of Ag-primed B cells within 3 days of their transfer into unimmunized recipient mice (**Fig. 2.4c**). A minor population (<7%) of Ag-primed Ig-Tg B cells proliferated in recipient mice (**Fig. 2.5a**). To avoid the confounding effect of proliferation, quantitative analysis of B cell numbers was performed on the unproliferated fraction of Ig-Tg cells normalized to cotransferred naïve control cells, which did not proliferate. This analysis indicated no progressive apoptosis of the Ag-pulsed B cells *in vivo* (**Fig. 2.5a**, **b**). The survival of Ag-primed B cells *in vivo* was independent of cognate or noncognate interactions with Th cells as indicated by similar persistence of Ag-primed B cells in αβ T cell deficient TCRα$^{-/-}$ recipient mice (**Fig. 2.4d**). Of note, similar proliferation of a small fraction of DEL-OVA-primed Ig-Tg B cells was observed in TCRα$^{-/-}$ recipient mice, indicating that proliferation was T-independent (**Fig. 2.5a**). In contrast to B cells primed with DEL-OVA

only once, continuous re-exposure of B cells to DEL-OVA in the absence of T cell help led to their progressive loss *in vivo* (**Fig. 2.4e**), as previously reported for MD4 B cells transferred into HEL-expressing recipient mice (106, 113).

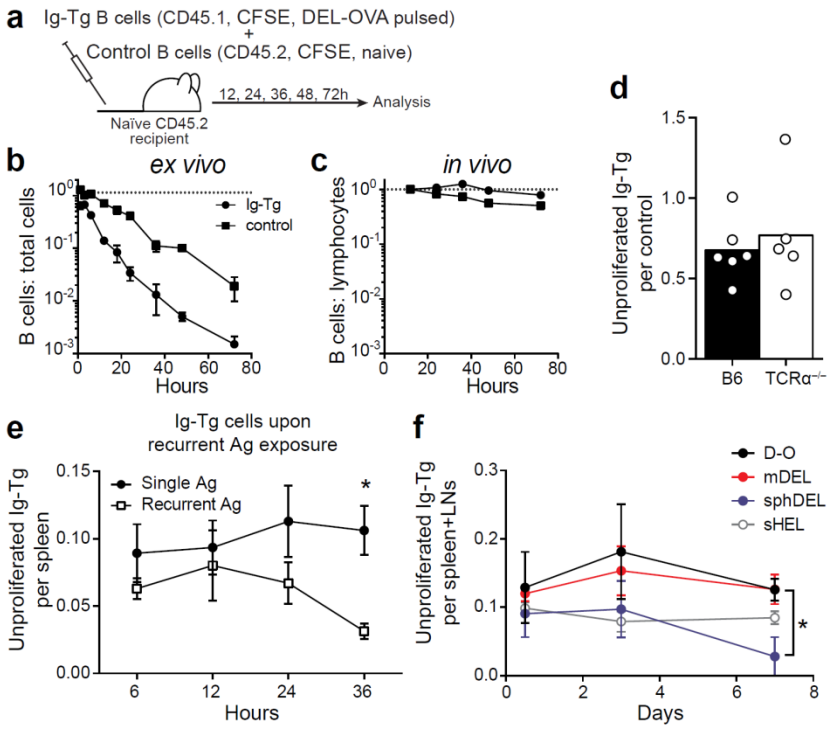

Figure 2.4. Ag-primed B cells do not undergo apoptosis in the absence of T cell help *in vivo*. **a**, Experimental outline for **c** and **d**. CFSE-labeled MD4 Ig-Tg B cells were pulsed *ex vivo* with 50 μg/mL DEL-OVA or DEL-OVA-Eα and co-transferred with CFSE-labeled naïve control B cells into unimmunized mice, then analyzed after various periods of time. **b**, **c**, Survival of Ag-pulsed Ig-Tg and control B cells in the absence of T cell help when cultured *ex vivo* (**b**, normalized to 0 hours, n=3 independent experiments) or *in vivo* in spleens of recipient mice (**c**, normalized to 12 hours, n=5 independent experiments). Data shown as mean ± SEM. **d**, Ratios of unproliferated Ig-Tg B cells to control B cells in the spleens of recipient wild type (B6) and TCRα$^{-/-}$ mice at 72 h.p.t. Data are normalized to the injected ratios of Ig-Tg to control B cells. Each symbol represents one mouse, bars at mean. n=2 independent experiments. **e**, Time-course analysis of DEL-OVA-pulsed (50 μg/mL) unproliferated Ig-Tg B cell numbers per spleen in unimmunized recipient mice injected (white dots) or not (black dots) with 50 μg DEL-OVA i.v. at 0, 12 and 24 h, normalized to the number of injected Ig-Tg cells. Data shown as mean ± SEM. n=2 independent experiments with 6 mice per condition. *p<0.05 (two-tailed Mann-Whitney test). **f**, Time-course analysis of DEL-OVA (50 μg/mL), mDEL (50 μg/mL), sphDEL (4x10^9 spheres/mL, 0.17 μg/mL DEL), and sHEL (1 μg/mL) pulsed unproliferated Ig-Tg B cell numbers per combined spleen and LNs in unimmunized recipient mice, normalized to the number of injected Ig-Tg cells. Data shown as mean ± SEM. n=2 independent experiments with 4 mice per condition. *p<0.05 between DEL-OVA pulsed and sphDEL pulsed Ig-Tg B cells (Kruskal-Wallis test with Dunn's post-test).

To test how Ag valency affects B cell survival *in vivo*, we first characterized the ability of various forms of Ig-Tg B cell Ags to downregulate BCRs *ex vivo*. DEL-OVA induced a similar amount of BCR crosslinking and receptor occupancy in Ig-Tg B cells as moderately multivalent DEL (mDEL) generated by oligomerization of biotinylated DEL with streptavidin. Both of these Ags induced Ig-Tg BCR crosslinking and internalization substantially more efficiently than monovalent forms of either HEL or DEL, but less efficiently than very highly multivalent polystyrene beads coated with DEL (sphDEL) (156) (**Fig. 2.5c**, **d**). Then, to determine whether Ag valency alters B cell survival following transient Ag acquisition, Ig-Tg B cells were pulsed with soluble Ag (sHEL), moderately multivalent Ag (DEL-OVA or mDEL), or highly multivalent Ag (sphDEL), and their persistence *in vivo* was monitored for 7 days. Axial, brachial, cervical, inguinal, and mesenteric LNs were collected in addition to spleens to account for recirculation of transferred cells. Although transient exposure to soluble or moderately multivalent Ags did not result in B cell disappearance from the secondary lymphoid organs within this time frame, transient exposure to highly multivalent Ag had a negative effect on B cell survival, a trend observed as early as 12 hours post transfer, with a significant decline in B cell number by 7 days (**Fig. 2.4f**, **Fig. 2.5e**). The observed decline in unproliferated sphDEL-pulsed B cell numbers was not due to their increased proliferation *in vivo* (**Fig. 2.4f**, **2.5f**).

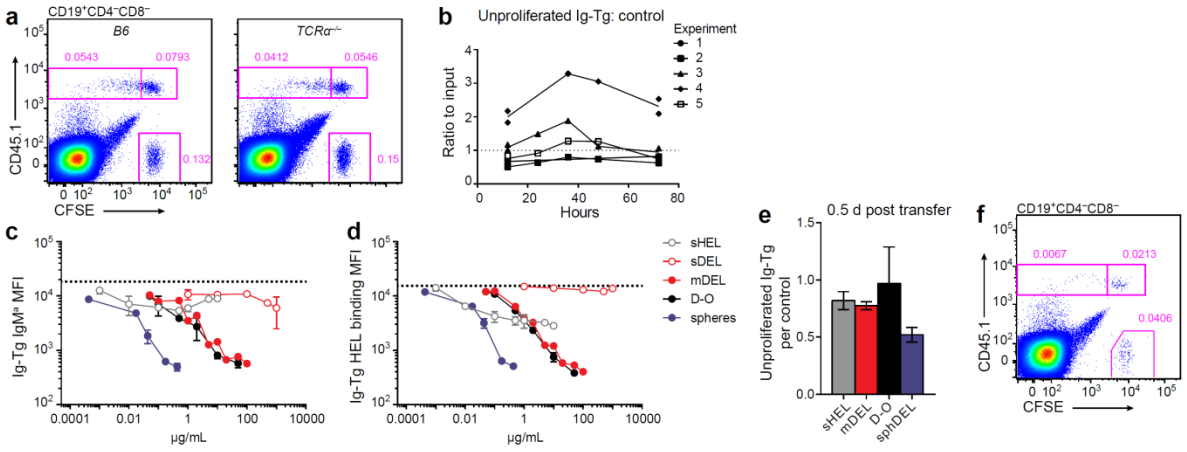

Figure 2.5. Ag-primed B cell gating strategy and dose response. a, **b**, Time-course analysis of DEL-OVA-pulsed (50 μg/mL) Ig-Tg MD4 B cells' survival in the spleens of unimmunized recipient mice. For experimental approach see **Fig. 2.4a**. **a**, Gating strategy for unproliferated ($CD45.1^+$ $CFSE^{high}$) and proliferated ($CD45.1^+$ $CFSE^{int/low}$) Ig-Tg and control ($CD45.1^-$ $CFSE^{high}$) B cells from the spleens of recipient mice. The examples shown correspond to 72 h post B cell transfer. [Legend continues next page].

[Fig. 2.5, cont'd.]
The left and right panels are from B6 and TCRα$^{-/-}$ recipient mice, respectively. **b**, Ratios of unproliferated Ig-Tg B cells to control B cells in the spleens of recipient mice, normalized to the injected ratio of Ig-Tg to control B cells. Each symbol represents one mouse. Data from n=5 independent experiments. **c**, **d**, Ig-Tg B cells' surface IgM^a (BCR) (**c**) and fluorescent HEL binding (**d**) following *ex vivo* pulsing with the indicated doses of Ags and 3 h culture. Data from n=2 independent experiments, shown as mean ± SEM. **e**, Ratios of unproliferated Ig-Tg B cells to control B cells in the spleens of recipient mice 12 h after pulsing with the indicated Ags, normalized to the injected ratio of Ig-Tg to control B cells. Data shown as mean ± SEM. n=2 independent experiments with 4 mice. **f**, Representative plot of unproliferated and proliferated Ig-Tg and control B cells gated as in (**a**) from the spleens of recipient mice 72 h after sphDEL pulse and transfer. Representative of n=2 independent experiments with 4 mice.

Ag-primed B cells return to a naïve-like state

Since we detected no significant apoptosis of B cells transiently primed with DEL-OVA in the absence of T cell help *in vivo*, we asked whether these B cells remained activated and capable of receiving T cell help. At 12 hours after their transfer into unimmunized recipient mice, Ag-primed B cells in the spleen were located predominantly at the borders between B cell follicles and T cell zones and had downregulated their surface IgM^a BCRs. By 24 hours, the cells re-upregulated their BCRs, downregulated surface expression of CCR7 receptors, and started to relocalize back into B cell follicles (**Fig. 2.6a–g**, **Fig. 2.7a**). Consistent with B cell inactivation, progressive downregulation of surface CD86 molecules and MHCII/antigenic Eα complex presentation by Ag-primed B cells were observed between 24 and 72 hours (**Fig. 2.6h–k**, **Fig. 2.7b**). CD86 and CCR7 downregulation at 3 and 7 days post transfer were also observed following transient Ig-Tg B cell priming with sHEL, mDEL, and sphDEL. To determine whether the kinetics of B cell inactivation may depend on the amount of Ag initially acquired, Ig-Tg B cells were pulsed *ex vivo* with various doses of DEL-OVA-Eα, and then characterized *in vivo* 6 and 24 hours later. Pulsing Ig-Tg B cells with concentrations of DEL-OVA-Eα up to ~2.5 μg/mL

resulted in a linear increase in surface presentation of antigenic peptide (**Fig. 2.6l**, **m**). Interestingly, while Ig-Tg B cells primed with all concentrations of DEL-OVA-Eα had decreased presentation of I-A^b/ Eα by 24 hours (**Fig. 2.6n**), those primed with lower amounts of Ag downregulated CD86 more quickly, suggesting that duration of B cell activation may depend on the dose of initially acquired Ag (**Fig. 2.6o**).

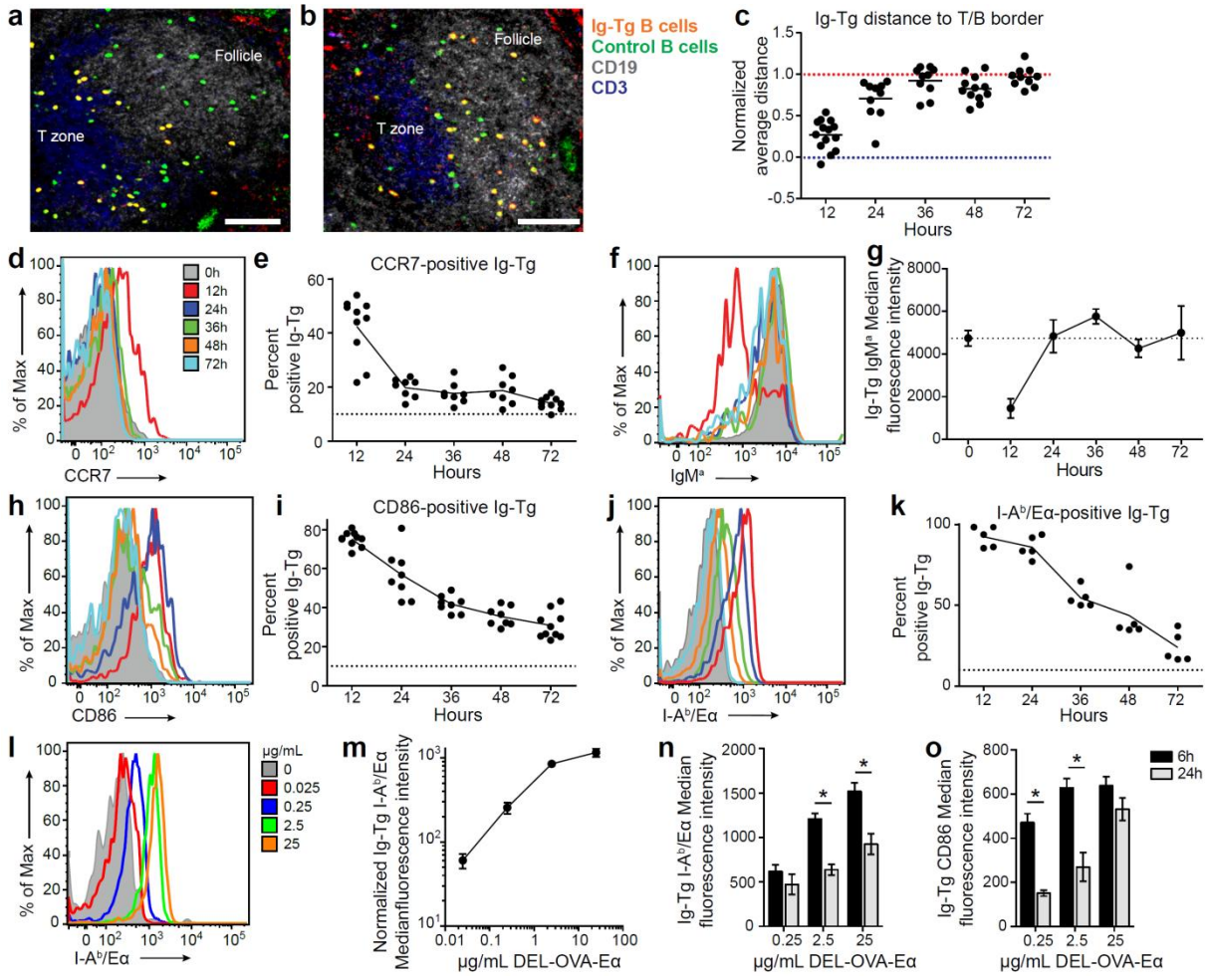

Figure 2.6. In the absence of T cell help Ag-primed B cells return to a quiescent state *in vivo*. **a**–**k**, Time-course analysis of DEL-OVA or DEL-OVA-Eα-pulsed (50 μg/mL) MD4 Ig-Tg and control B cells' localization (**a**–**c**) and surface marker expression (**d**–**k**) in the spleens of unimmunized recipient mice. For experimental set up see **Fig. 2.4a**. **a**, **b**, Representative confocal micrographs of spleen sections at 12 (**a**) and 24 (**b**) h.p.t of Ag-pulsed Ig-Tg cells. T/B border is at interface between T zone (blue) and follicle (white). Scale bars = 70 μm. **c**, Average distance of Ig-Tg cells to T/B border, normalized to average distance to the border of randomly distributed points in the follicle. Each dot represents one follicle and adjacent T/B border, line at mean. [Legend continues next page]

[Fig. 2.6, continued]
Blue line corresponds to cell distribution along T/B border, red line to random distribution in the follicles. See **Fig. 2.7 a**. n=3 independent experiments. **d**, **f**, **h**, **j**, Representative histographs of Ig-Tg B cells' surface expression of CCR7 (**d**), IgM^a BCR (**f**), CD86, (**h**), and I-A^b/Eα (**j**) at the indicated times after transfer into recipient mice. **e**, **i**, **k**, Percent of Ig-Tg cells staining positive for CCR7 (**e**), CD86 (**i**), and I-A^b/Eα (**k**). Positive gates defined at fluorescence brighter than 90% of control cells (dotted line at 10%), see **Fig. 2.7 b**. **g**, IgM^a Median fluorescence intensity. n=5 (**d**, **e**, **h**, **i**), n=3 (**j**, **k**), and n=2 (**f**, **g**) independent experiments. Each point corresponds to one mouse (**e**, **i**, **k**) or is shown as mean ± SEM for 6 mice (**g**). **l–o**, MD4 Ig-Tg B cells' antigenic peptide presentation (**l–n**) and CD86 surface expression (**o**) after pulsing with the indicated concentrations of DEL-OVA-Eα and transfer into naïve recipient mice. Surface I-A^b/Eα representative histograph (**l**) and median fluorescence intensity normalized to mock-pulsed cells (**m**) 6 h after transfer. Median fluorescence intensity of surface I-A^b/Eα (**n**) and CD86 (**o**) at 6 and 24 h after transfer. n=3 independent experiments, 5 mice per timepoint, shown as mean ± SEM (**m–o**). *p<0.05 (unpaired two-tailed Student's t test).

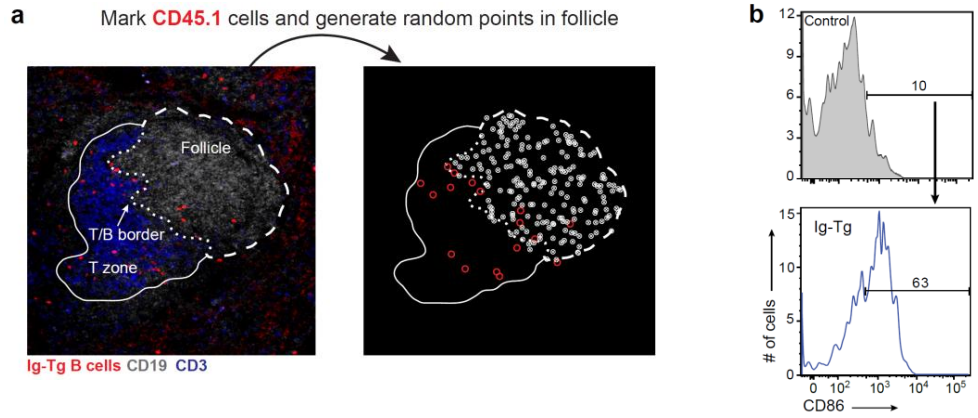

Figure 2.7. Analysis of B cell localization and activation markers. a, An illustration for quantitative analysis of Ag-pulsed Ig-Tg B cells' distance to the T/B border in spleen sections stained with CD19-, CD3-, and CD45.1-specific antibodies (see **Fig. 2.6a–c**). The follicle, T cell zone, and Ig-Tg B cells were marked in ImageJ and their coordinates read into a Matlab script which generated random points in the follicle and calculated the distance to the T/B border of each Ig-Tg B cell and randomly generated points. For each follicle the average distance to the T/B border of Ig-Tg B cells was then normalized to the average distance of randomly distributed points to derive normalized average distances in **Fig. 2.6c**. **b**, Determination of the "positive" gates for Ig-Tg B cells (See **Fig. 2.6 e**, **i**, **k**). The brightest 10% of control cells for a given marker were gated, and the gate applied to the Ig-Tg population; the fraction of the Ig-Tg population that fell within the gate was defined as positive.

Primed B cells are receptive to T cell help for 24–48 hours

To address the ability of Ag-primed B cells to receive delayed T cell help, Ig-Tg B cells pulsed with a high dose of Ag were transferred from primary recipients into OVA-immunized secondary recipient mice at various times, and their proliferation was assessed (**Fig. 2.8a**). As expected based on the kinetics of B cell inactivation, the majority of Ig-Tg B cells underwent proliferation when T cell help was available 12 hours following Ag priming. By 24 hours, only half of the cells could engage into the cell cycle, and by 48 hours most of the cells were unresponsive to T cell help (**Fig. 2.8b–e**). These data suggest that after transient acquisition of Ag, B cells have a 1-2 day window (or possibly less for lower doses of acquired Ag) for acquisition of T cell help before they return to a quiescent state.

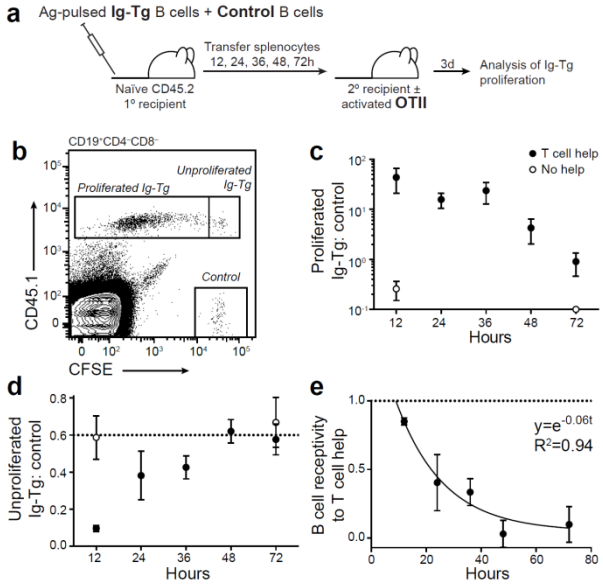

Figure 2.8. B cells lose receptivity to T cell help in parallel with phenotypic inactivation. Time-course analysis of Ag-pulsed Ig-Tg B cells' ability to receive T cell help and undergo proliferation *in vivo*. **a**, Experimental outline. CFSE-labeled MD4 Ig-Tg B cells were pulsed *ex vivo* with DEL-OVA or DEL-OVA-Eα and co-transferred with CFSE-labeled naïve control B cells into unimmunized mice. After various periods of time, splenocytes from recipient mice were analyzed and transferred to secondary recipient mice which had received OTII Th cells and been immunized i.p. with OVA in Ribi 3 d before. **b**, Gating strategy for proliferated and unproliferated Ig-Tg and control B cells. **c**, **d**, Ratios of proliferated (**c**) and unproliferated (**d**) Ig-Tg B cells to control B cells in preimmunized (filled circles) and control unimmunized (open circles) secondary recipient mice, normalized to the ratio of Ig-Tg to control B cells at the time of transfer. Data shown as mean ± SEM. n=5 independent experiments. **e**, B cell receptivity to T cell help calculated as $(1–F_I/F_C)$, where F_I and F_C are ratios of unproliferated Ig-Tg B cells to control cells in immunized and control secondary recipient mice, respectively. Data shown as mean ± SEM.

Primed and inactivated B cells can reenter humoral responses

To determine whether Ag-primed B cells that underwent inactivation were anergic or could reacquire Ag and T cell help and mount a productive humoral response *in vivo*, secondary recipient mice with inactivated Ag-primed Ig-Tg B cells were reimmunized with DEL-OVA (**Fig. 2.9a**). Three days after DEL-OVA immunization the majority of previously inactivated Ig-Tg B cells underwent proliferation (**Fig. 2.9b**, **Fig. 2.10a**) and by 6–7 days post immunization generated strong GC and PC responses, comparable to those of naïve Ig-Tg B cells, which had not previously encountered Ag (**Fig. 2.9c**, **d**). To eliminate the possibility that the GC and PC response was predominantly mounted by a small fraction of Ag-exposed Ig-Tg cells that underwent T-independent proliferation, $3–7x10^3$ unproliferated, Ag-primed and then inactivated or naïve Ig-Tg B cells were FACS-sorted from primary recipient mice 5 days after transfer and injected into secondary recipient mice, wherein their recruitment into the B cell response upon DEL-OVA administration was assessed. Unproliferated Ag-primed and then inactivated and naïve Ig-Tg B cells generated comparable GC and PC responses (**Fig. 2.9e**, **g**). Of note, under these experimental conditions, the magnitudes of the induced GC and PC responses were sensitive to the number of Ag-responsive B cells, suggesting that Ag-primed then inactivated and naïve B cells generated GC and PC responses with similar efficiency (**Fig. 2.9e–h**). While the possibility cannot be excluded that some Ig-Tg B cells remained residually activated and contributed to the observed GC and PC response, analysis of the sorted cells at the time of transfer suggested these cells would be very rare, as no more than 7% of the Ag-experienced population of Ig-Tg cells expressed higher amounts of CD86 than naïve control cells (**Fig. 2.10b**).

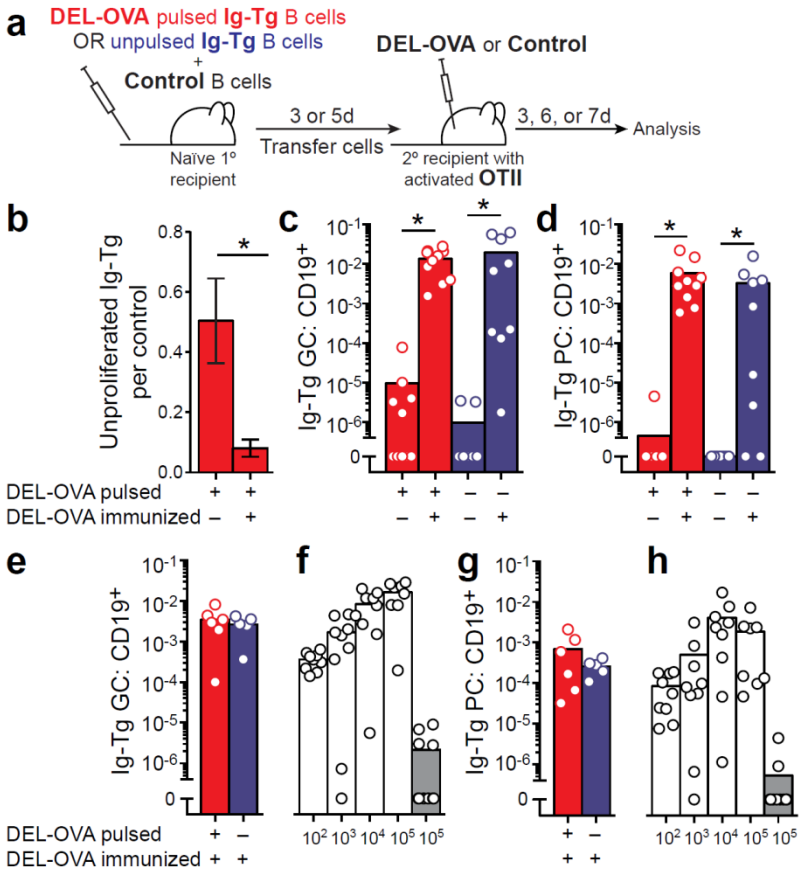

Figure 2.9. Inactivated B cells can re-acquire Ag and participate in the T dependent humoral immune response. Analysis of proliferation (**b**), GC (**c**, **e**, **f**) and PC (**d**, **g**, **h**) responses mounted by DEL-OVA-pulsed and then inactivated or naïve Ig-Tg B cells upon reacquisition of Ag. **a**, Experimental design. DEL-OVA-pulsed (50 μg/mL) or naïve MD4 Ig-Tg B cells were transferred into unimmunized mice. After 3 d (**b**–**d**) or 5 d (**e**, **g**) splenocytes (**b**–**d**) or FACS-sorted unproliferated Ig-Tg B cells (**e**, **g**) were transferred from primary to secondary recipient mice which had received OTII Th cells and been preimmunized i.p. with OVA in Ribi for 3 d. Immediately after Ig-Tg cell transfer, non-control secondary recipient mice were re-immunized with DEL-OVA in Ribi. **b**, Ratios of unproliferated Ig-Tg B cells to control B cells in non-reimmunized or DEL-OVA re-immunized secondary recipient mice, normalized to the input B cell ratios from primary recipients. Data from n=3 independent experiments with 5 mice per condition, shown as mean ± SEM. *, P<0.05 (two-tailed Mann-Whitney test). **c**–**e**, **g**, Participation in the GC (**c**, **e**) and PC (**d**, **g**) response by Ag-pulsed and then inactivated (red) or unpulsed naïve (blue) Ig-Tg B cells in DEL-OVA reimmunized or non-reimmunized secondary recipient mice 6 d (**c**, **d**) or 7 d (**e**, **g**) post re-immunization. **f**, **h**, GC (**f**) and PC (**h**) response participation 7 d.p.t. of the indicated number of unpulsed naïve Ig-Tg B cells to OVA-immunized recipient mice with OTII Th, either reimmunized with DEL-OVA (white) or non-reimmunized (grey). Ig-Tg B cell fractions of total splenic B cells are shown; each dot represents one mouse, bars at mean. **c**, **d**, Data from n=5 independent experiments with 10 mice (DEL-OVA pulsed Ig-Tg) or n=3 with 9 mice (unpulsed Ig-Tg). **e**, **g**, Data from n=3 experiments with 6 (pulsed Ig-Tg) or 5 (unpulsed Ig-Tg) mice. **f**, **h**, Data from n=2–3 independent experiments with 8–10 mice per condition. *p<0.05 (Kruskal-Wallis test with Dunn's post-test).

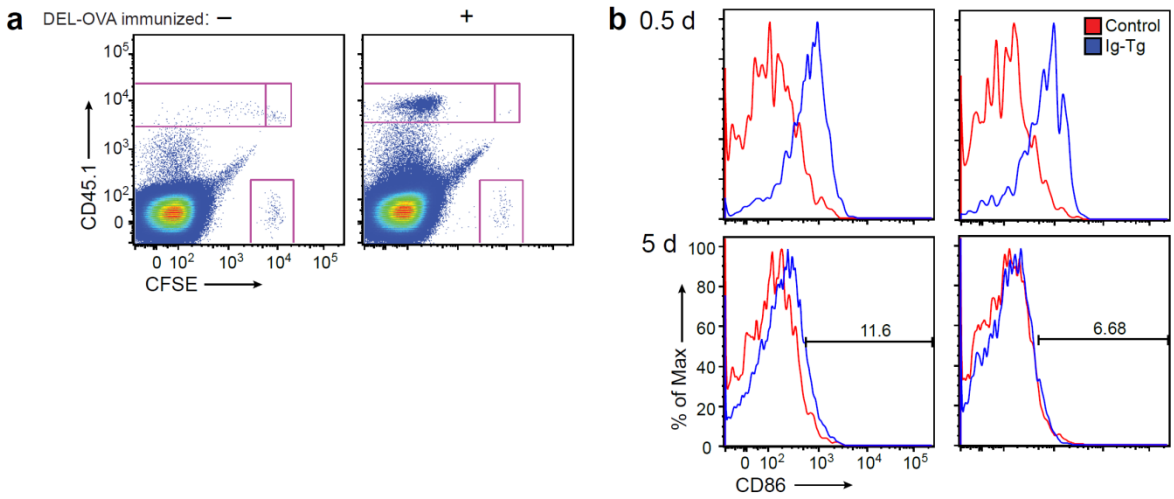

Figure 2.10. Inactivated B cells can proliferate after re-acquiring Ag and T cell help. a, Representative plots for unproliferated (CD45.1^{+} CFSE^{high}) and proliferated (CD45.1^{+} CFSE$^{int/low}$) Ig-Tg, and control (CD45.1^{-} CFSE^{high}) B cells from spleens of non-reimmunized (left) and DEL-OVA reimmunized (right) secondary recipient mice 3 days post reimmunization. Plots representative of n=3 independent experiments with 5 mice per condition**. b**, Representative CD86 histographs of Ig-Tg and control B cells 0.5 d.p.t. (top) and 5 d.p.t. (bottom). Two histographs are shown for each timepoint. The fraction of Ig-Tg B cells expressing higher CD86 than control at 5 d was estimated as the difference in percentage of Ig-Tg and control cells that fell within the gate defining the brightest 5% of control cells. Plots representative of n=3 independent experiments with 6 mice.

Discussion

In summary, the findings described above suggest that transient foreign Ag acquisition by B cells can be sufficient for their recruitment into the T-dependent humoral response and generation of GCs, memory B cells, and PCs *in vivo*. Previous *ex vivo* analysis of intracellular signaling and transcriptional regulation in B cells suggested that transient exposure to Ag may be sufficient to prime B cells to receive T cell help (116). Consistent with that, we find that *in vivo* in the presence of T cell help, B cells transiently primed with moderately multivalent Ag undergo proliferation and are recruited into the GC and class-switched memory response. They also generate a modest PC and class-switched antibody response. We observe similar results regardless of whether the numbers of Ag-pulsed Ig-Tg B cells and cognate Th cells are above or within the reported physiologic frequencies of Ag-specific endogenous cells (92, 123, 158). Based on these data we conclude that continuous or recurrent exposure to Ag is not required for

B cells' ability to acquire T cell help and proliferate or to be recruited into GC, memory, and PC responses *in vivo*. Moreover, B cells are recruited over a very broad range of Ag amounts acquired and level of antigenic peptides presented (**Fig. 2a–d**, **Fig. 2.6l**, **m**). In contrast, timely availability of T cell help is critical for recruitment of transiently Ag-primed B cells into the humoral response.

Our studies demonstrate that B cells transiently exposed to moderately-crosslinking Ags have a limited window of time *in vivo* to acquire T cell help and be recruited into the B cell response. Quantitative analysis suggests that B cells' ability to acquire T cell help and undergo proliferation *in vivo* decreases 2-fold within 24 hours of transiently acquiring of Ag, and is completely abolished between 36 and 48 hours. We show that *in vivo*, transiently Ag-primed B cells that do not get T cell help undergo sequential changes in localization and expression of surface markers that are consistent with their coordinated inactivation (**Fig. 2.11**). At 12 hours following transient exposure to Ag B cells localize to the border between the follicle and T cell zone and express high levels of costimulatory molecules and Ag-derived peptides, which are downregulated after B cells begin to migrate back into follicles by 24 hours.

Our studies also show that transiently Ag-experienced B cells are not rendered unresponsive to restimulation with Ag following functional and phenotypic inactivation. Transiently pulsed and then inactivated B cells have similar capacity as naïve B cells to join GC and PC responses *in vivo* upon re-exposure to Ag and T cell help. These data raise the possibility that B cells may go through multiple rounds of transient Ag acquisition followed by inactivation while waiting for T cell help.

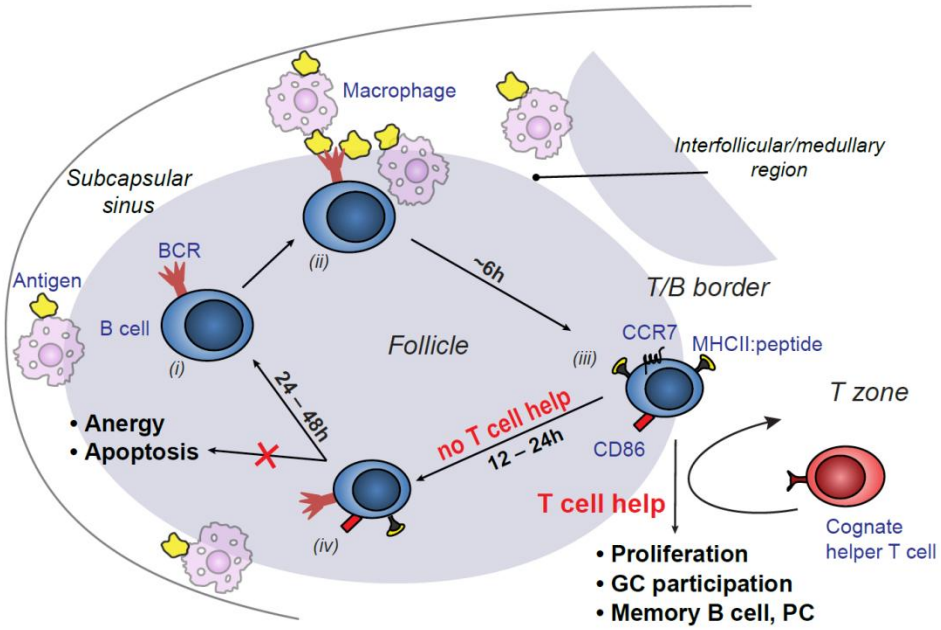

Figure 2.11. Proposed model of early Ag and T cell help acquisition events by B cells. The model summarizes the early events in large particulate Ag acquisition by B cells prior to generation of immune complexes and Ag transport onto follicular dendritic cells. Naïve B cells in the follicle (i) migrate to the subcapsular sinus and interfollicular regions of secondary lymphoid structures or medullary sinuses, where they can transiently acquire Ag (ii) and then leave. Within 6 h, they localize to the T/B border (iii) where, if they encounter T cell help within approximately 12–24h, they can proliferate and participate in the GC, memory, and PC responses. If T cell help is not acquired within this time, then B cells migrate back into the follicle and gradually downregulate expression of activation markers and Ag-derived peptide: MHCII complexes (iv). The inactivated B cells are not anergic and do not undergo apoptosis; rather they can acquire Ag again and have additional opportunities for recruitment into the T-dependent humoral immune response.

Surprisingly, we found that in the absence of productive T cell help, B cells transiently primed with saturating amounts of low to moderately crosslinking Ag do not undergo apoptosis *in vivo*, in contrast to their rapid death *ex vivo* (116). Of note, transient exposure to very highly multivalent particles does lead to B cell loss *in vivo*, consistent with the previously reported negative impact of very strong BCR signaling on B cell survival (153). While tonic CD40L signaling by non-cognate Th cells promotes survival of anergic B cells *in vivo*, our studies indicate that survival of B cells following transient Ag acquisition is independent of signals from cognate or non-cognate Th cells (160). Future studies should address which factors or combination of factors promote survival of transiently Ag-primed B cells *in vivo*.

In contrast to B cells transiently exposed to Ag, we detect progressive loss of Ag-specific B cells after 24 hours of recurrent exposure to Ag in the absence of T cell help *in vivo*. This is consistent with multiple studies demonstrating increased apoptosis and development of anergy in B cells continuously exposed to self-Ag (106, 113). While our findings challenge the postulate that Ag-experienced B cells must die in the absence of T cell help, they are consistent with the "time-honored hypothesis" which predicts that tolerance is induced by prolonged Ag receptor signaling in the absence of a second signal (100, 161). Future studies should address the molecular mechanisms which direct mature B cells' fate decisions between tolerance and quiescence based on the duration of their exposure to Ag.

Based on the *in vivo* kinetic analysis of B cell fate performed in this study, we speculate that duration of initial Ag acquisition by B cells may be an important factor affecting the efficiency and clonal diversity of the B cell response. Given the low frequency of Ag-specific Th cells during initiation of the primary immune response (10^{-5}–10^{-6}) and the time it takes migratory dendritic cells to promote Th cell proliferation, it is not unlikely that productive encounters between Ag-exposed B cells and cognate Th cells may take a few days (92-94). Continuous exposure of B cells to Ag may then lead to their apoptosis or anergy prior to acquisition of T cell help, limiting the number of B cell clones recruited into the immune response. Therefore, when T cell help is delayed, a transient mode of initial Ag acquisition (as observed for large particulate Ags deposited at restricted sites in secondary lymphoid organs) may favor survival of Ag-specific B cells *in vivo* and allow for subsequent opportunities to reacquire Ag and T cell help and be recruited into the T-dependent humoral immune response (**Fig. 2.11**). Consistent with that, large particulate Ags, which are initially sequestered from B cell follicles, promote more efficient humoral responses than soluble Ags (83, 162, 163). This is likely due to multiple factors, so future studies should investigate how the kinetics and localization of Ag acquisition by B cells affect the magnitude and breadth of the B cell repertoire entering T-dependent humoral responses.

Altogether, our results indicate that transient Ag acquisition by B cells may be sufficient for their recruitment into T-dependent B cell responses and that in contrast to continuous Ag exposure, transient acquisition of Ag does not always induce anergy or death in the absence of T cell help. Such a mechanism may allow B cells multiple opportunities to be recruited into the humoral immune response and favor a more clonally diverse population of responding B cells.

We suggest that the temporal dynamics of B cell exposure to Ag are an important factor for consideration in vaccine development, particularly for immunocompromised people who have a limited number or diversity of responding T cells (164-167).

Chapter 3. Antigen Acquisition Enables Newly Arriving B Cells To Enter Ongoing Immunization-Induced Germinal Centers

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Abstract

Modern vaccines must be designed to generate long-lasting, high-affinity, and broadly neutralizing Ab responses against pathogens. The diversity of B cell clones recruited into germinal center (GC) responses is likely to be important for the Ag-neutralization potential of the Ab-secreting cells and memory cells generated upon immunization. However, the factors that influence the diversity of B cell clones recruited into GCs are unclear. As recirculating naive Ag-specific B cells arrive in Ag-draining secondary lymphoid organs, they may join the ongoing GC response. However, the factors that limit their entry are not well understood, and it is not known how that depends on the stage of the ongoing follicular T cell and GC B cell response. In this article, we show that, in mice, naive B cells have a limited window of time during which they can undergo Ag-driven activation and join ongoing immunization-induced GC responses. However, preloading naive B cells with even a threshold-activating amount of Ag is sufficient to rescue their entry into the GC response during its initiation, peak, and contraction. Based on these results, we suggest that productive acquisition of Ag may be one of the main factors limiting entry of new B cell clones into ongoing immunization-triggered GC responses.

Introduction

A hallmark of T-dependent B cell responses is generation of germinal centers (GCs), which are important for the development of long-term high affinity humoral immunity (168, 169). GCs are anatomical substructures in B cell follicles that form around follicular dendritic cells (FDCs). GCs are seeded by Ag-activated B cells that have acquired cognate T cell help, proliferated, and differentiated into GC B cells. Within GCs, B cells undergo extensive

proliferation, somatic hypermutation of their B cell receptors (BCRs), and class-switching and compete for Ag deposited on FDCs and for help from follicular helper T cells (Tfh) (170). Tfh cells can drive GC B cells' affinity maturation by providing help preferentially to GC B cells that present more antigenic peptides in the context of MHCII, thus rescuing GC B cells from apoptosis and promoting their proliferation (133, 146). In parallel, follicular regulatory T cells (Tfr) fine-tune GCs by down-regulating the magnitude of the GC response and by preventing expansion of non Ag-specific B cell clones (76, 77). GC B cells then differentiate into long-lived plasma cells and class-switched memory B cells that harbor immunoglobulins and BCRs, respectively with higher affinity to foreign Ags (49, 171-173).

While generation of long-lived plasma cells and memory B cells is a prerequisite for development of long-term humoral immunity, the diversity of B cell clones that participate in GC responses may contribute to the breadth of antigenic epitopes recognized by effector cells and therefore to the pathogen neutralization potential of the response.

While previous studies suggested that GCs are formed by relatively few B cells, recent works unambiguously demonstrated that GCs are seeded by 50-200 B cell clones (119, 174-176). However, the ability of Ag-specific B cells to populate early GCs is variable. When T cell help is limiting, B cell clones with relatively low affinity to Ag are recruited into GCs less efficiently (54).

Preexisting GCs can also be populated by new B cell clones following a boosting immunization (120). However, which factors control or limit recruitment of new B cell clones into ongoing GCs over the course of an infection or following a primary immunization is not known. Naïve Ag-specific B cells' ability to enter preexisting "late" GCs is potentially limited by multiple factors, including limited availability of Ags to naive cells, competition with pre-existing GC B cells for Tfh cell help, differences in the helper functions of Tfh cells over time (73), and increased exposure of B cells to Tfr cells. In this study, we attempted to assess how the likelihood of new B cell recruitment into GCs depends on the stage (initiation, peak, or contraction) of the Tfh/Tfr and GC response.

Our study suggests that B cells that transiently acquire a low amount of Ag can enter GCs at all stages of the response. However, the ability of naïve B cells to undergo Ag-dependent activation and recruitment into the GC response drops by 6-10 days after a standard immunization. We suggest that the main factor limiting the entry of new B cell clones into GCs after a primary immunization may be the availability of Ag for sampling by the naïve B cell repertoire.

Materials and Methods:

Mice. B6 (C57BL/6) mice were purchased from Charles River Laboratory. B6- CD45.1 ($Ptprc^a$ $Pepc^b$/BoyJ) were purchased from the Jackson Laboratory. BCR transgenic HyHEL10 (154) and MD4 mice (104) were generously provided by Jason Cyster. HyHEL10 mice were crossed with UBC-GFP (004353) (Jackson Laboratory) and with B6- CD45.1 mice and maintained on the B6 background. MD4 mice were crossed with B6-CD45.1 and maintained on the B6 background. Recipient mice were 6–10 weeks of age. All mice were maintained in a specific pathogen free environment and protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Ag preparation. Duck eggs were purchased locally. Duck egg lysozyme (DEL) was purified as previously described (154). Ovalbumin (OVA) were purchased from Sigma. DEL was conjugated to OVA via glutaraldehyde cross-linking as previously described (154).

Immunization and adoptive transfer and immunization. Where indicated, recipient mice were preimmunized subcutaneously (s.c.) in the flanks and base of tail and into front foot pads (f.f.p.) with 100 μg OVA in Ribi (Sigma) or s.c. with 50 μg DEL-OVA in Ribi.

HyHEL10 B cells were enriched from donor mice by negative selection as previously described (134). Transient exposure to Ag was performed as previously described with an Ag dose slightly above the threshold required for B cell activation (**Fig. 2.3**). In brief, purified HyHEL10 B cells were incubated with 0.5 μg/mL DEL-OVA *ex vivo* for 5 minutes at 37 °C and washed three times with room temperature DMEM supplemented with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 2% FBS, 10 mM HEPES, 50 IU/mL penicillin, and 50 μg/mL streptomycin. About 5 $x10^4$ DEL-OVA-pulsed or naïve HyHEL10 B cells were then transferred into recipient mice. To study MD4 B cell activation, splenocytes from MD4 CD45.1 mice with a

known frequency of transgenic B cells were transferred into recipient CD45.2 mice either unimmunized or s.c. pre-immunized with DEL-OVA in Ribi.

Flow cytometery and cytokine staining. Single-cell suspensions from inguinal lymph nodes (ILNs) were incubated with biotinylated antibodies for 20 minutes on ice, washed twice with 200 µl PBS supplemented with 2% FBS, 1 mM EDTA, and 0.1% NaN_3 (FACS buffer), and then incubated with fluorophore- conjugated antibodies and streptavidin for 20 minutes on ice, and washed twice more with 200 µl FACS buffer. For cytokine staining, lymphocytes from ILNs of immunized mice were first resuspended in 10% DMEM (DMEM supplemented with 4.5 g/L glucose, L- glutamine and sodium pyruvate, 10% FBS, 10 mM HEPES, 50 IU/mL penicillin, and 50 µg/mL streptomycin) and then cultured in a CO_2 incubator, at 37^oC and stimulated with Cell Activation Cocktail (Biolegend) that contains PMA, ionomycin and Brefeldin A for 6 hours according to the manufacturer's instructions. For FoxP3 and cytokine staining the cells were permeabilized and stained using FoxP3 staining buffer set (eBioscience) according to the manufacturer's instructions. Cells were then resuspended in FACS buffer for acquisition. Data were acquired on a FACSCanto and analyzed using FlowJo (TreeStar).

Immunofluorescence. The following Abs/reagents were used for confocal immunofluorescent analysis: biotinylated anti-mouse IgD (11-26/SBA-1, SouthernBiotech), CD4-CF594 (RM4-5, BD biosciences), and streptavidin-Alexa Fluor 647 (Life Technologies) or BCL6-A647(clone K112-91, BD-Pharmingen) and IgMa-PE (clone DS-1, BD-Pharmingen). Brachial lymph nodes (BLNs) were harvested from recipient mice, fixed for 1 h in 1% paraformaldehyde in PBS on ice and washed with PBS. They were then blocked overnight in 30% sucrose, 0.1% NaN_3 in PBS, embedded in Tissue- Tek optimum cutting temperature compound, snap-frozen in dry ice and ethanol, and stored at -70 °C. Thirty micron cryostat sections were cut from the tissue blocks, affixed to Superfrost Plus microscope slides (Fisher), and stained first with biotinylated anti-IgD Abs and then with anti-CD4 Abs and streptavidin as previously described (177). Alternatively they were stained with anti-Bcl6 and anti-IgMa Abs. Confocal analysis of the sections was performed using Leica SP5 with argon and helium-neon lasers, 2-channel Leica SP spectral fluorescent PMT

detector, and a 20x oil-immersion objective with a numerical aperture of 0.7. Images were processed using Imaris (Bitplane).

Statistics. Statistical tests were performed as indicated using Prism 6 (GraphPad). Differences between groups not annotated by an asterisk did not reach statistical significance.

Results

In order to determine whether the ability of B cells to enter immunization-triggered GCs depends on the stage of the GC response, we first analyzed the kinetics of GC B cell and follicular T cell responses in the draining lymph nodes (LNs) of mice immunized with the protein Ag ovalbumin (OVA) in Ribi adjuvant (**Fig. 3.2A**, **B**, gating strategy). In unimmunized mice there were over 3 times as many Tfr as Tfh cells (**Fig. 3.1A-C**). By 6 days post immunization (d.p.i.) the number of Tfh cells significantly increased, reaching a 5:1 Tfh:Tfr ratio. (**Fig. 3.1A-C**). This was followed by expansion of GC B cells that peaked at 10-14 d.p.i. (**Fig. 3.1D**, **E**). At 14 d.p.i Tfh cell numbers and the Tfh/Tfr cell ratio started to decrease, followed by a substantial decline in GC B cell numbers by 21 d.p.i. (**Fig. 3.1A-E**). Based on the observed kinetics of the GC response and previously published data, GC seeding in the draining LNs is likely to occur between 3 and 6 d.p.i. (46, 178). This is followed by the peak of the GC response at 10 d.p.i. and its resolution after 14 d.p.i.

Previous studies indicated that Tfh cells' cytokine production and effector functions vary depending on the stage of GC response (73). To test whether these observations would hold under our selected immunization conditions, we assessed production of IL-21 and IFNγ cytokines by Tfh cells at various times following immunization with OVA in Ribi. Consistent with previous findings (73), we observed a trend for decreased production of IL-21 by Tfh cells during the later stages of the Tfh cell response (**Fig. 3.1 F-I**). Interestingly, we also found that, during Tfh cells' contraction phase, production of IFN-γ by Tfh cells increased significantly (**Fig. 3.1 F-H**, **J**). To summarize, Tfh cell frequency, production of cytokines, and the Tfh/Tfr cell ratio vary during the different stages of the immunization-induced GC reaction; these might affect recruitment and participation of new B cell clones in T-dependent responses.

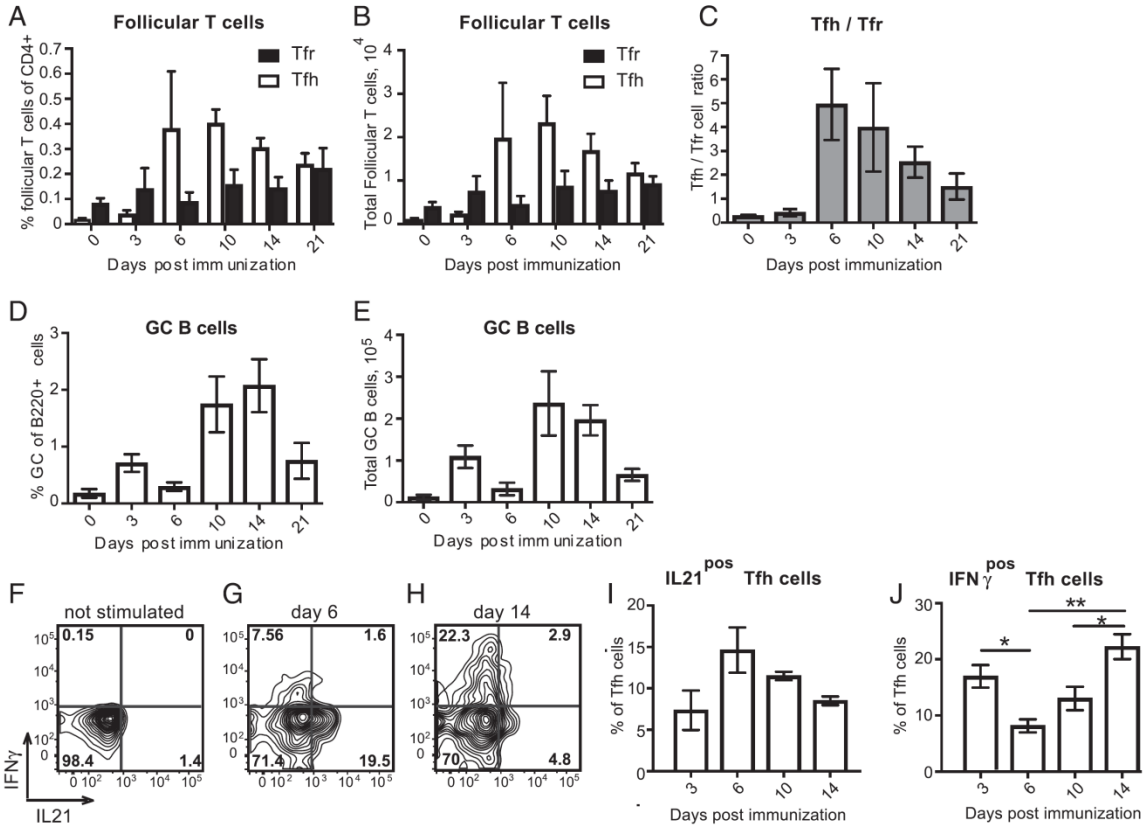

Figure 3.1. Kinetics of immunization-induced follicular T cell and GC B cell response. Flow cytometry analysis of follicular T cells and GC B cells in the ILNs of mice immunized s.c. with OVA in Ribi in the flanks, base of the tail, and into the front foot pads. (**A**) Frequencies of Tfh and Tfr cells among $CD4^+$ $B220^-$ $CD8^-$ cells. (**B**) Total numbers of Tfh and Tfr cells. (**C**) Tfh/Tfr cell ratios. (**D**) Frequencies of GC B cells among $B220^+$ $CD4^-$ $CD8^-$ cells. (**E**) Total numbers of GC B cells. (**F**–**J**) Analysis of IL-21 and IFN-γ production by Tfh cells at various times following immunization. Representative examples of flow cytometry cytokine staining of $FoxP3^-$ $CXCR5^{high}$ $PD1^{high}$ Tfh cells from ILNs of mice at 6 d.p.i. (**F**, **G**) and at 14 d.p.i. (**H**), following *ex vivo* stimulation with PMA and ionomycin (**G**, **H**) or no *ex vivo* stimulation. (**F**) Fraction of Tfh cells that upregulate production of IL-21 (**I**) or IFN-γ (**J**) following *ex vivo* stimulation. Data are from n=3 or 4 independent experiments, with 1–3 mice per experiment. Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ordinary one-way ANOVA with Tukey multiple-comparison test.

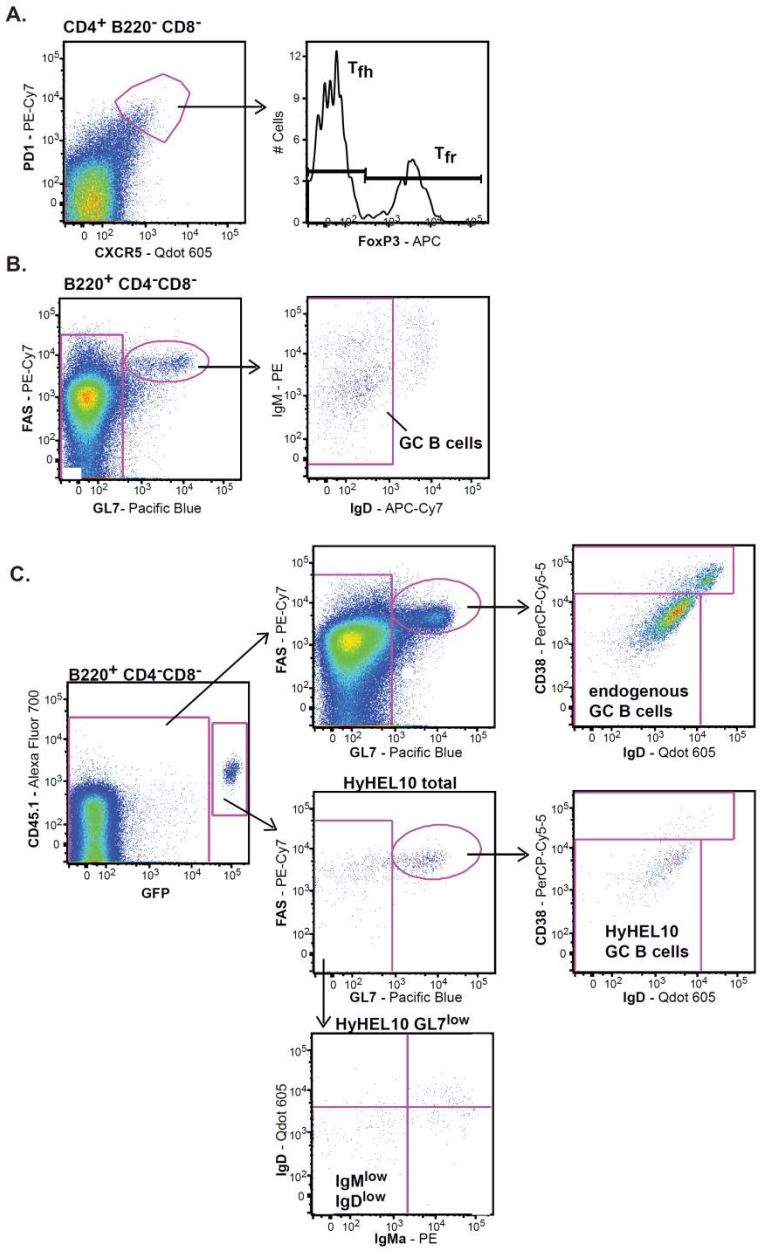

Figure 3.2. Gating strategy for follicular T cells, GC B cells and class-switched GL7^{low} B cells. (**A**, **B**) Flow cytometry gating strategy for endogenous Tfh and Tfr cells (**A**) and GC B cells (**B**) utilized for analysis presented in **Fig. 3.1**. **C**, Flow cytometry gating strategy for total, GC, and GL7^{low} class-switched HyHEL10 B cells utilized for analysis presented in **Figs. 3.2**, **3.3**, **and 3.4**.

To address whether B cells that acquire the same amount of Ag have a substantially different ability to enter GCs depending on the phase of the GC response we made use of our *ex vivo* pulsing experimental strategy (**Fig. 3.3A**). Transgenic HyHEL10 B cells (154) that have B

cell receptors (BCR) specific to duck egg lysozyme (DEL) were incubated *ex vivo* for 5 minutes with DEL chemically conjugated to OVA (DEL-OVA) at a concentration only slightly above HyHEL10 B cells' activation threshold (**Fig. 2.3**). The unbound Ag was then washed off and HyHEL10 B cells were transferred into mice preimmunized with OVA in Ribi. While DEL-OVA pulsed HyHEL10 B cells could not reacquire their cognate DEL Ag *in vivo*, they could present preacquired OVA antigenic peptides in the context of MHCII molecules for recognition by OVA-specific Th cells *in vivo*. Our previous study demonstrated that Ag-pulsed B cells transferred into recipient mice preimmunized with OVA for 3 d undergo proliferation, differentiate into GC B cells, and participate in histologically defined GCs *in vivo* and that their ability to enter the B cell response is critically dependent on the acquisition of the Ag-linked OVA for presentation to activated Th cells (**Fig. 2.1**).

To assess B cell recruitment into an immunization-driven GC response at its various stages, fifty thousand DEL-OVA pulsed HyHEL10 B cells were transferred into recipient mice at 3, 6, 10 and 14 days after immunization with OVA or, for control, into unimmunized mice (**Fig. 3.3A**). These times correspond to the initiation, peak and contraction phases of the Tfh cell response (**Fig. 3.1A-C**).

At 4 days after the transfer of Ag-pulsed HyHEL10 B cells, we observed their expansion in the draining LNs of all OVA-immunized recipient mice (**Fig. 3.3B**). Based on the $GL7^{high}$ FAS^{high} IgD^{low} $CD38^{low}$ phenotype (**Fig. 3.2C**), comparable numbers of HyHEL10 B cells differentiated into GC B cells in all OVA-immunized, but not in control recipient mice at 4 days after transfer (**Fig. 3.3E**; **Fig. 3.4**, **black bars**). At 6 days post transfer the numbers of HyHEL10 GC B cells either slightly increased (when transferred at 3, 6 d.p.i.) or stayed the same (at 10, 14 d.p.i.) (**Fig. 3.3F**; **Fig. 3.4**, **gray bars**). In all cases, HyHEL10 GC B cell numbers started to decline by 8 days after B cell transfer, as expected due to their inability to reacquire their cognate Ag DEL within GCs and compete with OVA-specific endogenous GC B cells (**Fig. 3.3G**; **Fig. 3.4**, **white bars**). Some of the Ag-pulsed HyHEL10 B cells also differentiated into $GL7^{low}$ class-switched B cells, which made up 20-40% of total HyHEL10 B cells by 6-8 days after their transfer (**Fig. 3.3B-D**, **H–J**). Few, if any, HyHEL10 plasma cells were found (**data not shown**).

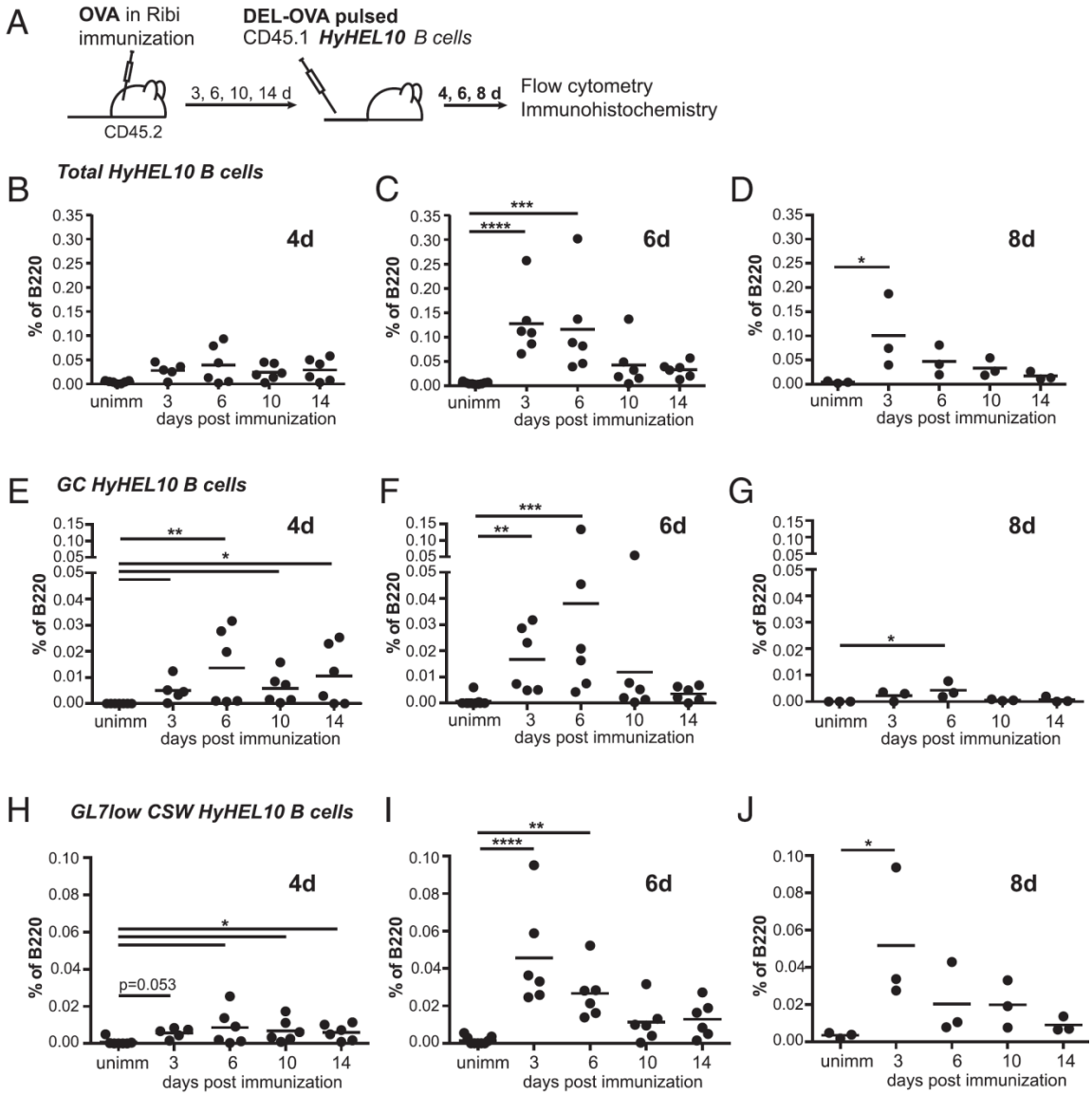

Figure 3.3. Ag-pulsed HyHEL10 B cell recruitment into B cell response during initiation, peak and resolution of the immunization-induced GC response. (**A**) Experimental scheme. A total of 5×10^4 HyHEL10 B cells, pulsed *ex vivo* with 0.5 μg/ml DEL-OVA, was transferred into unimmunized control mice or mice that were immunized with OVA in Ribi s.c. and in the front foot pads 3, 6, 10, or 14 d earlier. Flow cytometry analysis of ILNs was performed at 4, 6, and 8 d post-HyHEL10 B cell transfer. Frequencies of total HyHEL10 B cells (**B**–**D**) and $GL7^{high}$ FAS^{high} IgD^{low} $CD38^{low}$ HyHEL10 B cells (**E**–**G**) and $GL7^{low}$ IgM^{low} IgD^{low} HyHEL10 B cells (**H**–**J**) among $B220^+$ $CD4^-$ $CD8^-$ cells at 4 d (**B**, **E**, and **H**), 6 d (**C**, **F**, and **I**) and 8 d (**D**, **G**, and **J**) following the transfer. Data are from three to five independent experiments. Each dot represents one mouse. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $***p \leq 0.0001$ versus unimmunized control, Kruskal–Wallis test, with Dunn multiple-comparison test.

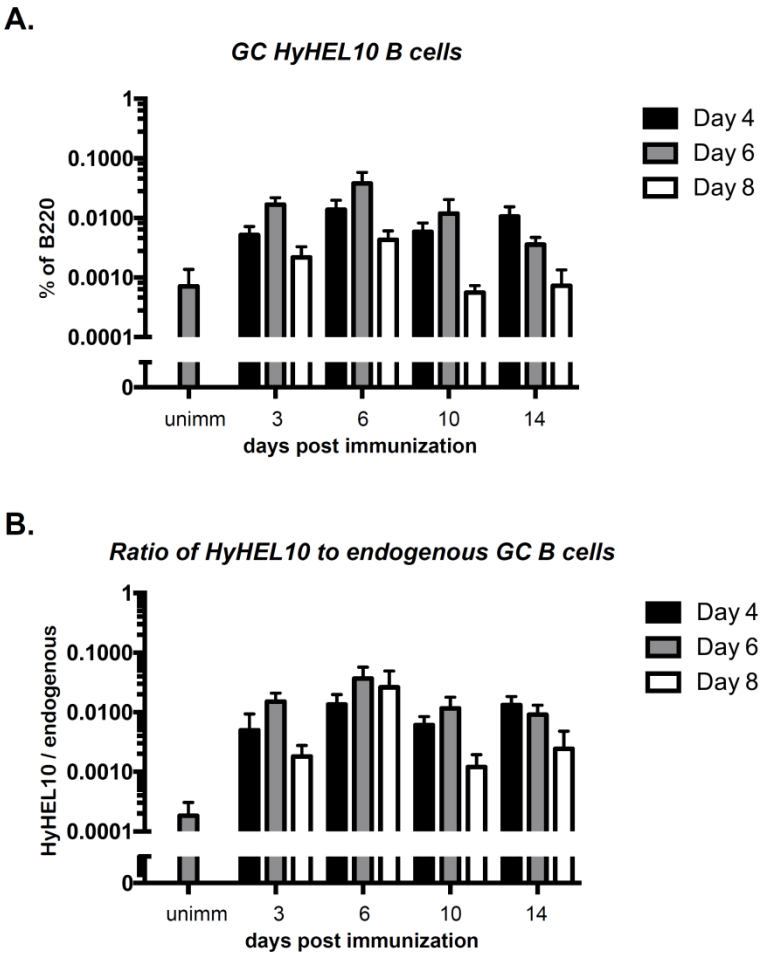

Figure 3.4. Ag-pulsed HyHEL10 B cell recruitment into GC responses during initiation, peak and resolution of the immunization-induced GC response. $GL7^{high}$ FAS^{high} IgD^{low} $CD38^{low}$ HyHEL10 GC B cells displayed as a fraction of $B220^{+}$ cells (**A**) and their ratio to endogenous GC B cells (**B**) at 4d (black bars), 6d (gray bars) and 8d (white bars) following transfer of DEL-OVA-pulsed HyHEL10 B cells into unimmunized mice or mice preimmunized with OVA in Ribi for 3, 6, 10 and 14 days. Data are from 3-5 independent experiments, shown as mean ± SEM. In **A**, data is combined from **Fig. 3.3 E–G**.

The presence of HyHEL10 B cells in the GCs of OVA-immunized, but not control mice, was confirmed by immunofluorescent analysis of draining LNs regardless of the time of transfer following immunization (**Fig. 3.5**, HyHEL10 B cells express GFP and are detected as green). The majority of GC resident HyHEL10 B cells also expressed Bcl6 (**Fig. 3.5B**; see examples 1, 4, 7, 10, 11). Of note, HyHEL10 B cells were also found in other regions of B cell follicles with some of them IgM^{a} positive (**Fig. 3.5B;** examples 2, 5) and some class-switched (**Fig. 3.5B;** examples 3, 6, 9).

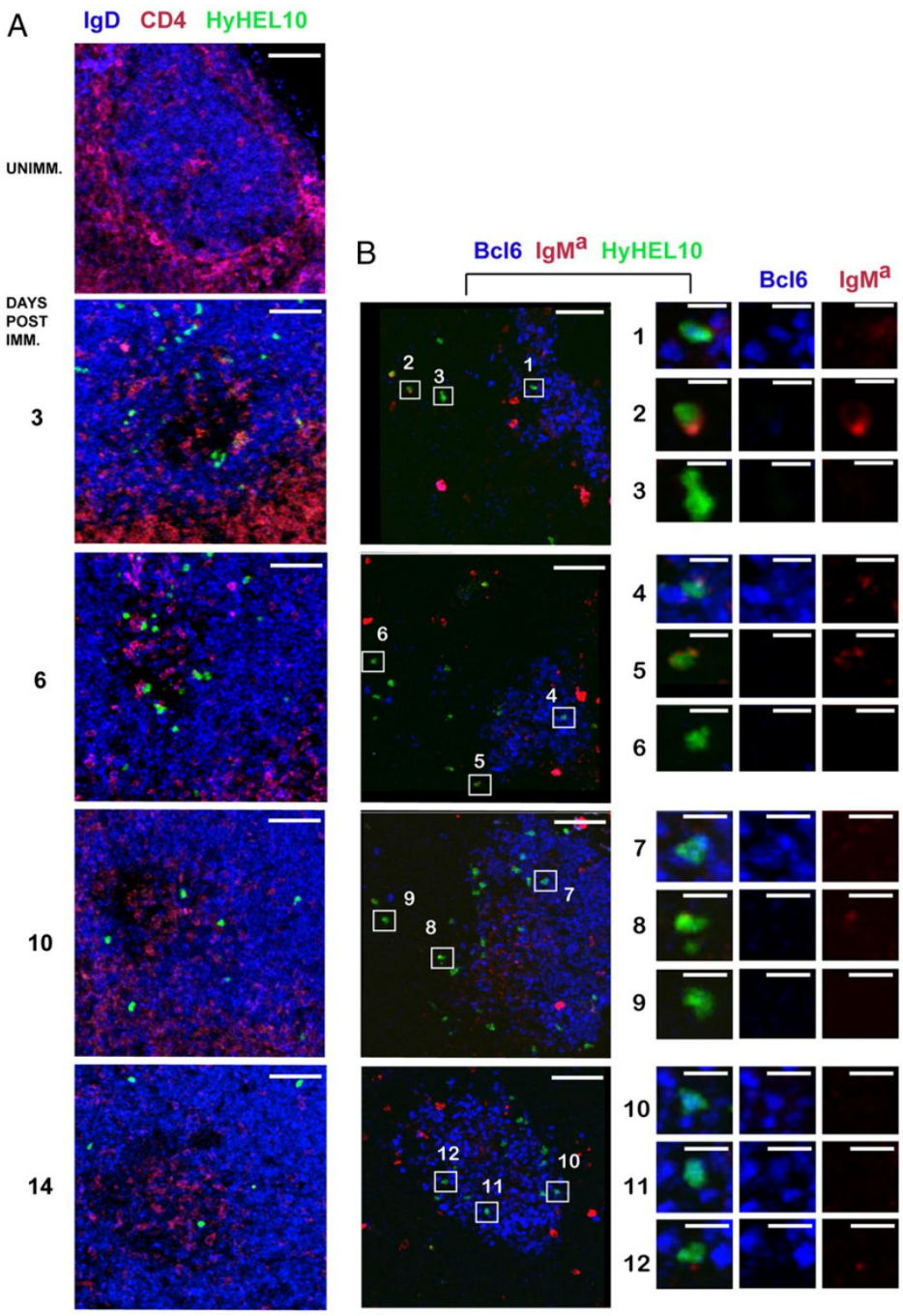

Figure 3.5. Ag-pulsed HyHEL10 B cells can enter GCs during the initiation, peak, and resolution of the immunization-induced GC response. (**A** and **B**) Examples of DEL-OVA pulsed HyHEL10 B cells' anatomical positioning relative to GCs. Confocal immunofluorescent analysis of 14–20-μm-thick sections from brachial LNs of mice that were immunized or not with OVA in Ribi 3, 6, 10, or 14 d before receiving DEL-OVA pulsed HyHEL10 B cells; slides were analyzed at 6 d post transfer. GFP-expressing HyHEL10 B cells are shown in green. Sections were stained with fluorescently conjugated anti-CD4 (red) and anti-IgD (blue) Abs (**A**) or IgM^a (red) and Bcl6 (blue) (**B**). (**B**) Right panels show a zoomed-in view of the HyHEL10 cells boxed in the left panels. In (**A**), IgD^{low} areas represent GCs. [Legend continues next page]

[Fig. 3.5, continued]
In (**B**), Bcl6^{+} cells are GC B cells; IgM^{a-} HyHEL10 B cells are CSW. Red staining not colocalized with GFP^{+} HyHEL10 cells is likely due to nonspecific binding of IgM^{a} to other cells, presumably to macrophages and FDCs. Data are representative of n=3 independent experiments, with one mouse per experiment. Scale bars: 50 μm (**A** and **B**, left panels), 10 μm (**B**, right panels).

Altogether these results indicate that at various stages of the OVA-immunization induced endogenous follicular T cell/GC response, acquisition of relatively small amounts of DEL-OVA Ag by newly arriving HyHEL10 B cells is sufficient for their recruitment into the GC and class-switched GL7^{low} memory B cell responses in OVA-draining lymph nodes. However, when Ag-pulsed B cells are transferred at the peak and resolution phases of the Tfh/GC response (at 10 and 14 d.p.i.), their subsequent accumulation as GC and class-switched GL7^{low} B cells at 6 and 8 days after transfer is reduced.

To test the ability of naïve B cells to enter the GC response during its various stages, naïve HyHEL10 B cells were transferred into mice immunized with their cognate Ag DEL-OVA in Ribi adjuvant at 0, 3, 6, 10 and 14 d.p.i (**Fig. 3.6A**). While HyHEL10 B cells transferred into recipient mice prior to or 3 days after the immunization mounted a vigorous GC B cell response, their ability to form GC B cells began to decline when they were transferred 6 days following the immunization and completely disappeared at later times (**Fig. 3.6B**, **white bars**). Naïve HyHEL10 B cells also formed substantially smaller class-switched B cell responses when transferred into DEL-OVA immunized mice with a 10-14 day delay (**Fig. 3.6C**, **white bars**). However, HyHEL10 B cells pulsed with a low dose of Ag (0.5 μg/ml of DEL-OVA) formed GC B cells and class-switched GL7^{low} cells when transferred into DEL-OVA immunized mice even at the peak and prior to contraction phases of the GC response, similarly to Ag-pulsed cells transferred into OVA-immunized mice (**Fig. 3.6B**, **C**, **gray bars**, **Fig. 3.3E–J**).

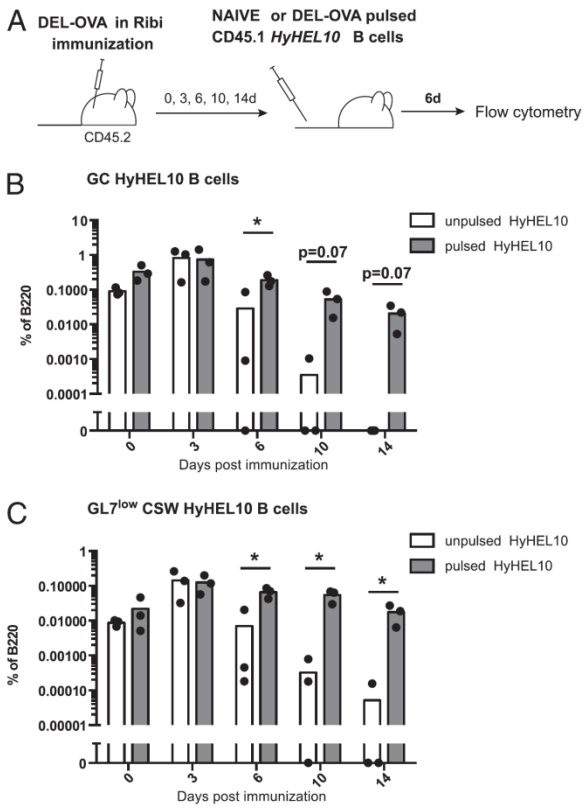

Figure 3.6. Recruitment of naïve and DEL-OVA-pulsed HyHEL10 B cells into B cell response during initiation, peak and resolution of GC response in DEL-OVA immunized mice. (**A**) Experimental scheme. A total of 5×10^4 HyHEL10 B cells (naive or pulsed *ex vivo* with 0.5 μg/ml DEL-OVA) was transferred into mice immunized s.c. with DEL-OVA in Ribi 0, 3, 6, 10, or 14 d earlier. Flow cytometry analysis of ILNs was performed at 6 d post-HyHEL10 B cell transfer. Frequencies of unpulsed or DEL-OVA–pulsed HyHEL10 $GL7^{high}$ FAS^{high} IgD^{low} $CD38^{low}$ (**B**) and HyHEL10 $GL7^{low}$ IgM^{low} IgD^{low} (**C**) B cells among $B220^+$ $CD4^-$ $CD8^-$ cells. Data are from n=3 independent experiments. Each point represents one mouse. *$p < 0.05$, multiple *t* tests with the Holm–Sidak correction for multiple comparisons.

To test whether B cells have a limited window of time to undergo Ag-driven activation following a standard protein immunization, we utilized BCR transgenic B cells from MD4 mice that have the same specificity to DEL as HyHEL10 B cells. While MD4 B cells can not undergo class-switching as HyHEL10 B cells, they are better for enumeration of Ag-specific B cells by flow cytometry because they constitute the majority (>95%) of B cells in MD4 mice. Splenocytes from CD45.1 MD4 mice were transferred into recipient CD45.2 mice preimmunized with DEL-OVA in Ribi for 3, 6 and 10 days or into unimmunized control mice. One day later, Ag-driven activation of the recently arriving MD4 B cells was assessed in the ILNs of

preimmunized mice based on their upregulation of surface CD69 (**Fig. 3.7A**). We found that many MD4 B cells transferred at 3 days post immunization underwent activation (**Fig. 3.7B**, **C**). In contrast, transferred non-transgenic CD45.1 B cells did not upregulate CD69 in ILNs of DEL-OVA mice preimmunized for 3 days (**Fig. 3.7D**). The fraction of MD4 B cells that underwent Ag-dependent activation and upregulated CD69 significantly decreased between 3 and 10 days following DEL-OVA immunization (**Fig. 3.7B**, **C**). Based on these observations and the findings described above, we conclude that following immunization with standard protein Ag in Ribi adjuvant, Ag-driven activation becomes the predominant factor limiting the entry of new Ag-specific B cells into the ongoing GC B cell response.

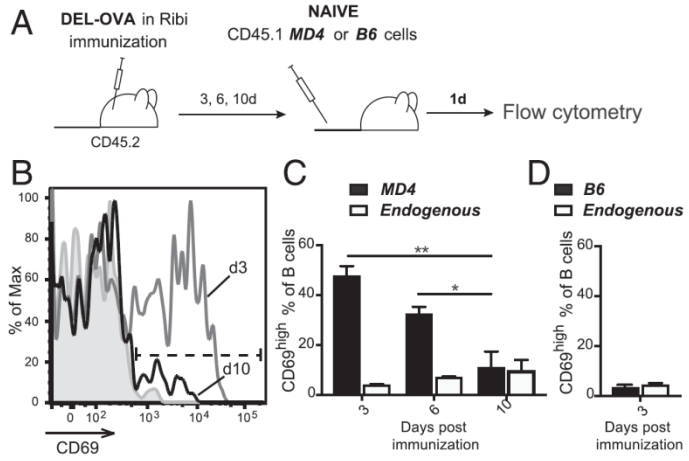

Figure 3.7. Ag-dependent activation of B cells at various times post immunization. (**A**–**D**) Upregulation of CD69 by naive MD4 or B6 B cells transferred into DEL-OVA–preimmunized mice. (**A**) Experimental scheme. A total of 2–10 × 10^5 naive CD45.1 MD4 B cells was transferred into mice immunized with DEL-OVA in Ribi s.c. 3, 6, or 10 d earlier or into unimmunized control mice. Similar numbers of CD45.1 B6 B cells were transferred into mice immunized with DEL-OVA in Ribi s.c. 3 d earlier. Flow cytometry analysis of ILNs was performed 1 d post–B cell transfer. (**B**) Representative flow cytometry analysis of MD4 B cell CD69 surface staining after their transfer into unimmunized mice (shaded graph) or mice immunized 3 d (gray line) or 10 d (black line) earlier. (**C**) Frequencies of $CD69^{high}$ MD4 B cells ($B220^+$ $CD4^-$ $CD8^-$ $CD45.1^+$ $CD45.2^-$; black bars) and endogenous B cells ($B220^+$ $CD4^-$ $CD8^-$ $CD45.1^-$ $CD45.2^+$; white bars). Data from n=3 independent experiments, with one mouse per experiment. (**D**) Frequencies of $CD69^{high}$ $B220^+$ $CD4^-$ $CD8^-$ $CD45.1^+$ $CD45.2^-$ B6 B cells that were transferred at 3 d post–DEL-OVA immunization (black bars) and $CD45.1^-$ $CD45.2^+$ endogenous B cells (white bars). Data are from two independent experiments, with four recipient mice. Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ordinary one-way ANOVA, Tukey multiple-comparison test.

Discussion

While the cellular and molecular mechanisms of GC B cell affinity maturation and their differentiation into memory B cells and plasma cells have been analyzed in a great level of detail, the factors that control clonal diversity of B cell responses are much less understood. In this work, we addressed which factors may limit the access of new Ag-specific B cell clones into GC and memory B cell responses following a standard protein/adjuvant immunization. First, we asked whether the ability of B cells uniformly exposed to the same small dose of cognate Ag to enter GC responses would be different during the initiation, peak, and resolution of the follicular T cell / GC B cell response. Our studies suggest that the initial expansion of Ag-experienced B cells *in vivo* and the generation of GC B cells do not strongly depend on the phase of the follicular T cell/GC response. We also confirmed histologically that B cells can enter GCs during various phases of follicular T cell/GC response. However, when Ag-exposed B cells enter the GC response during the peak or contraction phase their subsequent expansion as GC B cells and their differentiation into memory-like GL7low class-switched B cells is reduced. We speculate that the observed trend may be explained by the decreased ability of Tfh cells to support proliferation and survival of both older and newer GC B cell clones possibly due to the decrease in Tfh cell numbers and altered cytokine-mediated activity and/or the relative increase in the contribution of Tfr cells that negatively impact GC sustainability (73, 76, 77).

We then assessed the ability of naïve Ag-specific B cells to enter the GC response at various times following immunization with their cognate Ag. We found that in contrast to B cells that pre-acquired a small amount of Ag, naïve B cells' ability to enter both the GC and $GL7^{low}$ CSW B cell responses drops by 6-10 days following immunization. That correlated with significantly decreased ability of Ag-specific B cells to undergo "early" activation. This outcome is consistent with naïve B cell access to Ag becoming relatively limited *in vivo* after a few days following the immunization, suggesting it is a critical factor for new Ag-specific B cell clones' recruitment into the GC response. The fact that boosting immunizations promote recruitment of new Ag-specific B cell clones into preexisting GCs is consistent with this hypothesis (120).

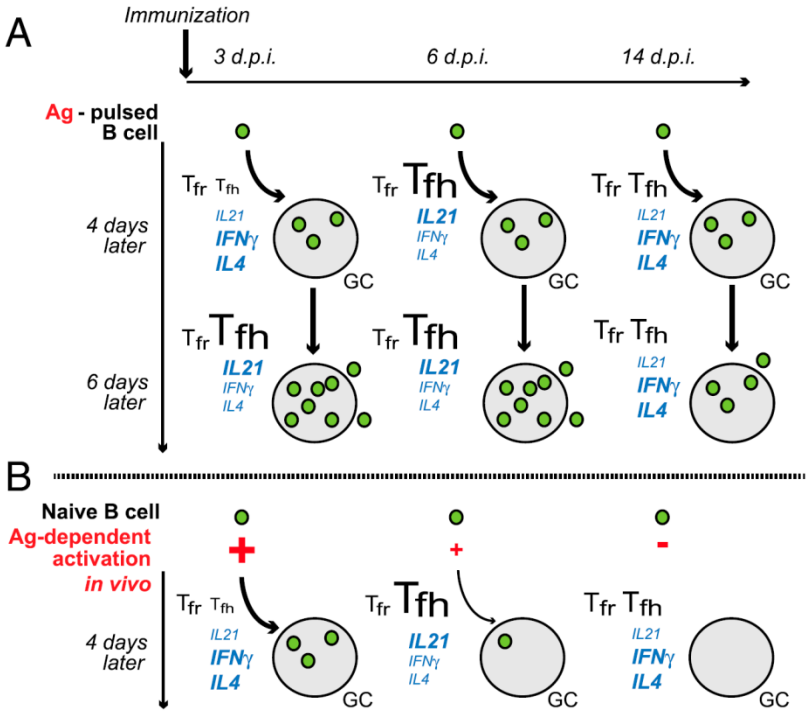

Figure 3.8. Model of new B cell entry into the GC response at various times after immunization. (**A**) B cells exposed to a threshold-activating dose of Ag and cognate T cell help can enter the GC response with comparable efficiency at various times after immunization, including the initiation, peak, and resolution phases of the follicular T cell response. However, their subsequent expansion in GCs and formation of memory cells are reduced during the Tfh/GC resolution phase, possibly due to their decreased exposure to Tfh cell–produced IL-21 and/or increased repression from Tfr cells. (**B**) Following immunization, naive Ag-specific B cells have a limited window of time during which they can enter the GC response. This is determined by Ag-dependent activation of the naive B cells entering into Ag-draining LNs. In mice immunized s.c. with DEL-OVA in Ribi, Ag-dependent activation and entry into GCs starts to decrease at 6 d.p.i.

One possibility is that over time naïve B cells' access to Ag becomes more restricted. Formation of immune complexes leads to Ag redistribution onto FDCs (179). While Ags persist on FDCs for prolonged periods of time supporting the GC response, a previous study indicated progressive loss in naïve B cells' ability to acquire Ags from FDCs over time (91). Gradual degradation of Ag and formation of GCs around FDCs may partially limit access of naïve B cells to FDC-presented Ags. Steric hindrance by the antibodies generated during the early B cell response in some cases may additionally limit Ag acquisition by newly arriving B cell clones (180). Further studies are necessary to discriminate between these possibilities and thus decipher

factors controlling recruitment of new B cell clones into ongoing immunization-induced GCs. Future studies should also address whether various adjuvants or Ag administration protocols affect the window of time in which new B cells may enter ongoing GCs following immunization. Dissecting these factors may be important for improving the diversity of B cell clones entering GCs following vaccinations and thus increasing the chances of generating broadly neutralizing antibody responses.

Chapter 4. Transiently Antigen Primed B Cells Can Generate Multiple Subsets of Memory Cells

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Abstract

Memory B cells are long-lived cells that generate a more vigorous response upon recognition of antigen (Ag) and T cell help than naïve B cells and ensure maintenance of durable humoral immunity. Functionally distinct subsets of murine memory B cells have been identified based on isotype switching of BCRs and surface expression of the co-stimulatory molecule CD80 and co-inhibitory molecule PD-L2. Memory B cells in a subpopulation with low surface expression of CD80 and PD-L2 are predominantly non-isotype switched and can be efficiently recruited into germinal centers (GCs) in secondary responses. In contrast, a CD80 and PD-L2 positive subset arises predominantly from GCs and can quickly differentiate into antibody-secreting plasma cells (PCs). Here we demonstrate that single transient acquisition of Ag by B cells may be sufficient for their long-term participation in GC responses and for development of various memory B cell subsets including CD80 and PD-L2 positive effector-like memory cells that rapidly differentiate into class-switched PCs during recall responses.

Introduction

Long term humoral immunity is critical for protection from many pathogens and is elicited by most successful vaccines. Upon primary infection or immunization, a small population of Ag-specific B cells becomes activated and expands after acquiring T cell help (49). Some of these expanded clones differentiate into memory B cells, which circulate and can be rapidly recruited into the humoral immune response upon reacquisition of Ag and T cell help (51, 123, 181). Other activated B cells are recruited into GCs, in which they undergo somatic hypermutation of their BCRs and higher affinity clones are selected based on their ability to

acquire Ag and T cell help (57). Memory B cells and antibody-secreting PCs differentiate from GC B cells after iterative rounds of mutation andselection, although memory cells are thought to undergo less stringent affinity-based selection compared to PCs (131, 132). Memory B cells can be most broadly defined as B cells that have been activated by Ag and persist in its absence (125, 126). A number of recent studies have demonstrated that functionally distinct subsets of murine memory B cells can be identified phenotypically, either by expression of an isotype-switched or unswitched BCR (51, 123, 124) or by expression of combinations of the co-stimulatory and co-inhibitory molecules CD80 and PD-L2 (29, 127). While memory B cells that form prior to GCs are predominantly IgM positive, the majority of memory B cells that differentiate from GCs are class-switched (51, 52, 123). Studies employing isotype switching as a marker of functional heterogeneity found that class-switched memory B cells are more "effector memory-like," that is, they differentiate more rapidly into PCs upon reencountering Ag and T cell help, whereas IgM memory cells were more "naïve-like" in that they were more predisposed to participate in secondary GCs prior to differentiating into PCs (123, 124). Studies using CD80 and PDL2 to differentiate subsets of memory cells identified at least three functionally distinct memory subsets, defined as double negative (DN, $CD80^{-}PD\text{-}L2^{-}$), single positive (SP, $CD80^{-}PD\text{-}L2^{+}$), and double positive (DP, $CD80^{+}PD\text{-}L2^{+}$). Isotype-switched and unswitched memory B cells are present in all three subpopulations, but the DN population consists of predominantly IgM^{+} B cells, whereas the SP and DP populations are comprised of progressively more class-switched cells and demonstrate increasing propensity to differentiate quickly into PCs. While DN cells are the most naïve-like, DP cells are thought to originate predominantly from GCs (29, 52, 127).

Previously we found that single transient Ag acquisition is sufficient for B cell recruitment into immune responses when T cell help is available, including their participation in histologically-defined GCs and differentiation into PCs and memory B cells (**Fig. 2.1**). Interestingly, compared to GC and PC responses, the short-term memory B cell response appeared least affected by the dose of transiently acquired Ag or reacquisition of Ag by the participating B cells. However, in that study memory cell subsets and class-switching were not quantitatively assessed. In addition, the memory B cell response was only analyzed out to 21 days, while later timepoints were not examined. Therefore, whether transient acquisition of Ag by B cells is sufficient for their differentiation into the memory B cell subsets described above is not known, and whether memory cells generated by B cells that transiently acquire Ag persist in

the periphery is unclear. In the work described below we demonstrate that single transient acquisition of Ag may be sufficient for long-term participation of B cells in GC responses and for development of various memory B cell subsets including DP and class-switched cells that can quickly differentiate into PCs during a recall response.

Materials and Methods

Mice. C57BL/6 (B6) and Ptprc^{a} Pepc^{b}/BoyJ (B6-CD45.1) mice were purchased from the Jackson Laboratory. BCR transgenic Hy10 mice (C57BL/6 background) (154) were generously provided by Jason Cyster. Hy10 mice were crossed with B6-CD45.1 mice and maintained on this background. Donor and recipient mice were 6–12 weeks of age. All mice were maintained in a specific pathogen free environment and protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Ag preparation. Duck eggs were locally purchased and duck egg lysozyme (DEL) was purified as previously described (154). BSA and OVA were purchased from Sigma, and DEL was conjugated to OVA via glutaraldehyde cross-linking as previously described (154).

Immunization and adoptive transfer. Male recipient mice were immunized s.c. in the flanks and base of tail with 50 μg BSA, OVA, or DEL-OVA emulsified in CFA (Sigma), prepared according to the manufacturer's directions. Where indicated, recipient mice were reimmunized with 50 μg DEL-OVA emulsified in IFA (Sigma), prepared according to the manufacturer's directions.

Hy10 B cells were enriched from male and female donor mice by negative selection as previously described (134). For transient exposure to Ag, purified Hy10 B cells were incubated with DEL-OVA *ex vivo* for 5 minutes at 37°C, washed four times with room temperature DMEM supplemented with 4.5 g/L glucose, L-glutamine and sodium pyruvate, 2% FBS, 10 mM HEPES, 50 IU/mL of penicillin, and 50 μg/mL of streptomycin, and transferred i.v. to recipient mice.

Flow cytometery. Single-cell suspensions from spleens or draining inguinal lymph nodes (dLNs) were incubated with biotinylated antibodies (**Table 1**) for 20 minutes on ice, washed twice with 200 μl PBS supplemented with 2% FBS, 1 mM EDTA, and 0.1% NaN_3 (FACS buffer), incubated with fluorophore-conjugated antibodies and streptavidin (**Table 1**) for 20 minutes on ice, washed twice more with 200 μl FACS buffer, and resuspended in FACS buffer

for acquisition. For intracellular staining, surface-stained cells were fixed and permeabilized for 20 minutes on ice with BD Cytofix/Cytoperm buffer, washed twice with 200 μl BD Perm/Wash buffer, incubated with for 20 minutes on ice with fluorophore-conjugated antibodies (**Table S1**), followed by two washes with 200 μl Perm/Wash buffer, and resuspended in FACS buffer for acquisition. Data were acquired on a FACSCanto or LSRFortessa and analyzed using FlowJo (TreeStar).

Statistics. Statistical tests were performed as indicated using Prism 6 (GraphPad). Differences between groups not annotated by an asterisk did not reach statistical significance. No blinding or randomization was performed for animal experiments, and no animals or samples were excluded from analysis.

Results

To determine the ability of B cells to differentiate into various subpopulations of memory B cells *in vivo* after a single transient acquisition of Ag, and to define how their development and persistence over time depends on the dose of initially acquired Ag and reacquisition of Ag *in vivo*, we used an experimental approach similar to that described before (**Fig. 4.1A**) (177). Purified Hy10 B cells specific for avian lysozyme (104, 154) were pulsed *ex vivo* for 5 minutes with either a saturating (50 μg/mL) or threshold activating (0.5 μg/mL) concentration of the moderate affinity Ag duck egg lysozyme (DEL) (157) fused to ovalbumin (DEL-OVA) and the unbound Ag was then washed off. 10^5 Ag-pulsed Hy10 B cells were transferred into recipient mice, which had been s.c. immunized with OVA in CFA three days earlier to activate endogenous OVA-specific helper T cells. Under these conditions, DEL-OVA-primed B cells could not reacquire cognate Ag *in vivo*, but could digest pre-acquired OVA, present OVA-derived peptides, and make cognate interactions with activated OVA-specific Th cells (**Fig. 4.1A**, **upper panel**). As a positive control, Hy10 B cells pulsed with a saturating concentration of DEL-OVA as described above or which remained unpulsed were transferred to DEL-OVA immunized mice, in which Ag could be re-acquired (**Fig. 4.1A**, **middle panel**). As negative controls, Hy10 B cells were pulsed with a saturating dose of DEL-OVA and transferred to recipient mice preimmunized with the irrelevant Ag BSA. In the BSA-immunized mice no cognate Th cell help would be available for DEL-OVA pulsed Hy10 B cells at the time of their transfer (**Fig. 4.1A**, **lower panel**).

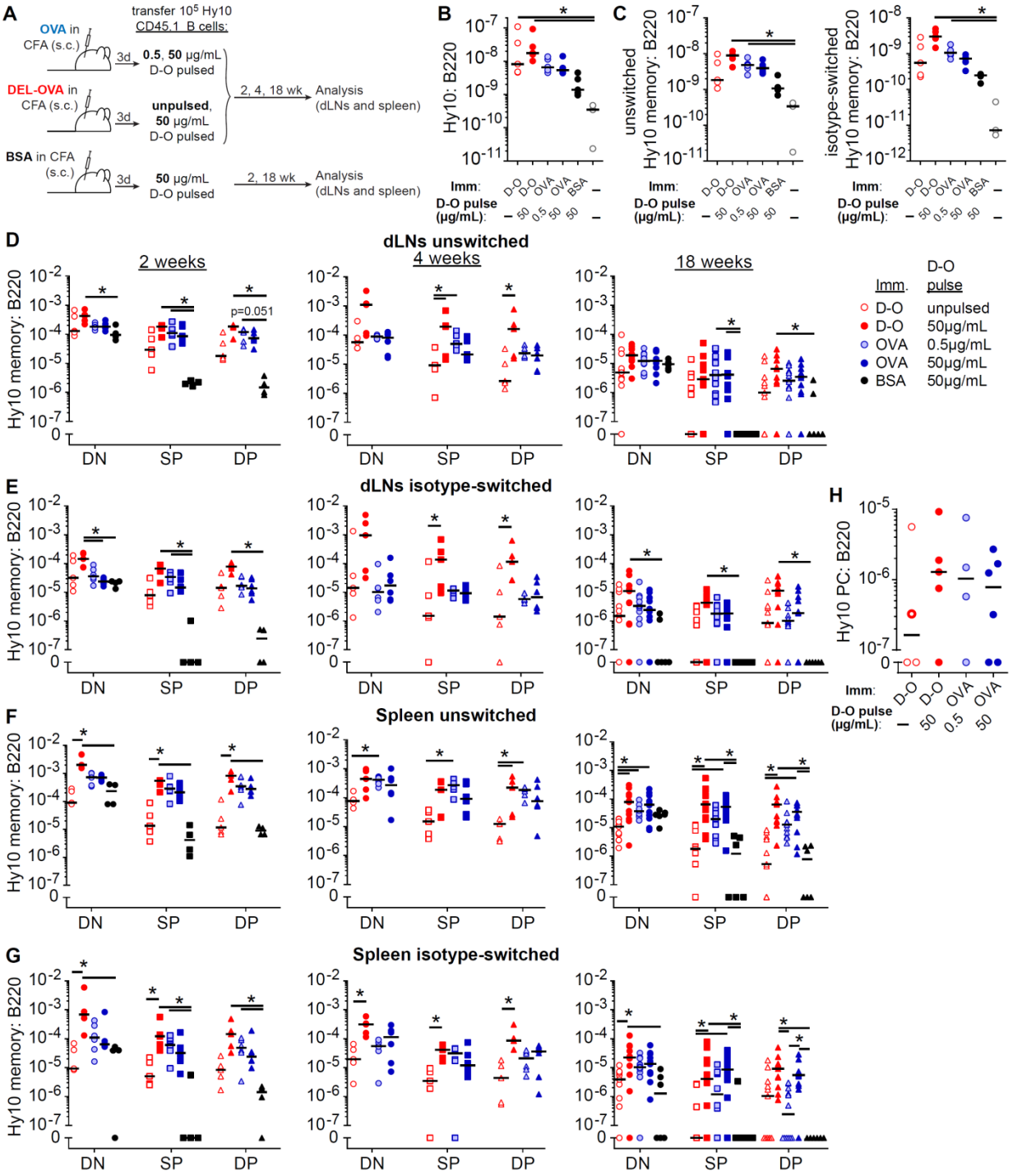

Figure. 4.1. Single acquisition of threshold activating amount of Ag enables generation and persistence of memory B cells *in vivo*. A, Experimental outline. Unpulsed Hy10 B cells or Hy10 B cells pulsed *ex vivo* for 5 min with 0.5 or 50 µg/mL DEL-OVA were transferred into recipient mice s.c. preimmunized with OVA, DEL-OVA, or BSA in CFA. **B**, **C**, Expansion of Hy10 cells in recipient mice 2 weeks after transfer. Total (**B**) and GL7$^-$ (**C**) unswitched (left), and isotype-switched (right) Hy10 cells from LNs of recipient mice, shown as fraction of B220 normalized to the number of Hy10 cells transferred. [Legend continues next page]

[Fig. 4.1, continued]
n=2 experiments with 3-6 mice. **D–G**, Memory B cell responses of unpulsed (open symbols) and 50 μg/mL (filled symbols) or 0.5 μg/mL (shaded symbols) DEL-OVA pulsed Hy10 B cells in draining inguinal LNs (dLNs, **D**, **E**) and spleens (**F**, **G**) of OVA (blue symbols), DEL-OVA (red symbols), and BSA (black symbols) immunized recipient mice 2 weeks (left panels), 4 weeks (middle panels) and 18 weeks (right panels) after transfer. DN, SP, and DP subpopulations gated as in **Fig. 4.2A**, **C** and shown as ratio to total $B220^{+}CD4^{-}CD8^{-}$ singlet lymphocytes. **H**, Hy10 PC recall response in dLNs 3 days after secondary s.c. immunization with 50 μg DEL-OVA in IFA, 18 weeks after initial transfer of Hy10 cells. For 2 and 4 weeks and recall, n=2 experiments with 4–6 mice per condition; for 18 weeks, n=4 experiments, 6-12 mice per condition. Each symbol represents one mouse, line at median. Symbol with thicker line denotes DEL-OVA immunized recipient of naïve Hy10 cells in which unswitched PCs were recovered; all other recovered Hy10 PCs were class-switched. *$p<0.05$ (Kruskal-Wallis test with Dunn's post-test between naïve and each immunized condition (**B**, **C**) and all conditions at each timepoint (**D–H**). Differences between groups not annotated by an asterisk did not reach significance.)

We analyzed development of memory B cells by Hy10 cells in the dLNs and spleens of recipient mice at 2, 4, and 18 weeks after B cell transfer. Memory Hy10 B cells were identified as $CD4^{-}CD8^{-}$ $B220^{high}$ $GL7^{low}$ CD45.1 cells (**Fig. 4.2A**). Under all tested conditions the majority of $GL7^{low}$ Hy10 B cells were confirmed to express high levels of CD38 at 2 and 18 weeks, consistent with a memory or naïve B cell phenotype (**Fig. 4.2B**), (51). Using the gating strategy outlined in **Fig. 4.2C** we then identified unswitched ($IgM^{+}IgD^{+/lo}$) and class-switched ($IgM^{-}IgD^{-}$) $GL7^{low}$ B cells and further subcategorized them into double negative (DN, $CD80^{-}PD\text{-}L2^{-}$), single positive (SP, $CD80^{-}PD\text{-}L2^{+}$), and double positive (DP, $CD80^{+}PD\text{-}L2^{+}$) subsets as described before (29, 127). The gating was defined based on DEL-OVA pulsed Hy10 B cells from dLNs of DEL-OVA immunized mice relative to naïve Hy10 B cells from unimmunized mice (**Fig. 4.2C**).

We first examined development of memory B cells by Ag-pulsed Hy10 B cells in the OVA, DEL-OVA and BSA-immunized mice shortly after their transfer (**Fig. 4.1A**). At two weeks after transfer, Hy10 B cells expanded (**Fig. 4.1B**) and formed $GL7^{low}$ memory cells in the dLNs of recipient mice under all conditions, exceeding numbers of naïve Hy10 cells recovered from unimmunized recipient mice (**Fig. 4.1C**). Surprisingly, frequencies of DN class-switched and unswitched $GL7^{low}$ Hy10 cells were almost as high in BSA immunized recipients as in OVA immunized recipients, in which cognate T cell help was available (**Fig. 4.1D**, **E**, **left panels**), and the numbers recovered from dLNs were similar (**Fig. 4.3A**, **B**, **left panels**). Higher frequencies and numbers of DN switched and unswitched $GL7^{low}$ cells were observed in DEL-OVA

immunized recipients in which Hy10 cells pulsed with a large dose of Ag could reacquire Ag *in vivo* (left panels, **Fig. 4.1D**, **E** and **Fig. 4.3A**, **B**).

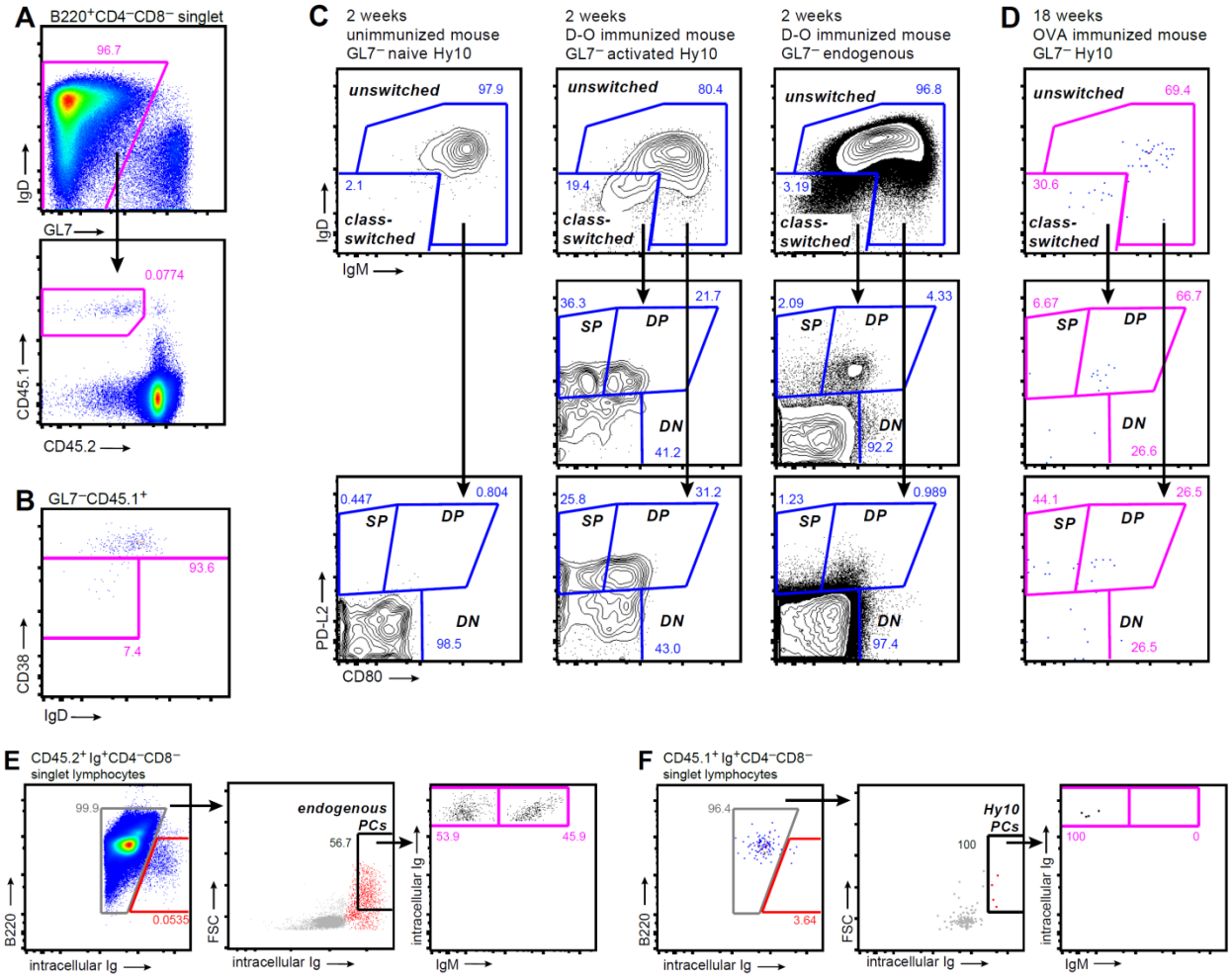

Figure 4.2. Memory B cell subset and PC gating strategies. A, Hy10 memory B cell gating strategy. Representative of n=2-4 experiments with 4-12 mice per condition. 2 week timepoint shown from dLNs. **B**, CD38 staining of GL7$^-$ Hy10 cells in dLNs. Representative of n=2 independent experiments with 4-6 mice at 2 and 18 weeks after transfer. 2 week timepoint shown. **C**, Class-switching and memory subpopulation gating. For recovery of high numbers of Hy10 naïve and memory cells for memory subpopulation gating, 5x10^6 unpulsed or 1x10^6 DEL-OVA pulsed Hy10 B cells were transferred to naïve and DEL-OVA immunized recipient mice, respectively. Draining LNs from DEL-OVA immunized and peripheral LNs from unimmunized recipients were analyzed 2 weeks after transfer. GL7$^-$ Hy10 (left, middle panels) and endogenous (right panels) B cells were gated as in **A**. Representative of n=2 independent experiments with 3-4 mice. **D**, Example of memory subpopulation gating from 18 week timepoint. Representative of n=4 independent experiments with 6-12 mice. **E**, **F**, Plasma cell gating strategy for endogenous (**E**) and Hy10 (**F**) cells. PCs were identified as B220^{lo} intracellular Ig^{hi} cells (left panels, red gates) that were larger and stained more brightly for intracellular Ig than B220$^+$ cells (middle panels, black gates). Ig$^+$B220$^+$ cells (grey gates) shown for comparison. Class switched PCs were defined based on intracellular IgM staining (lower panels).

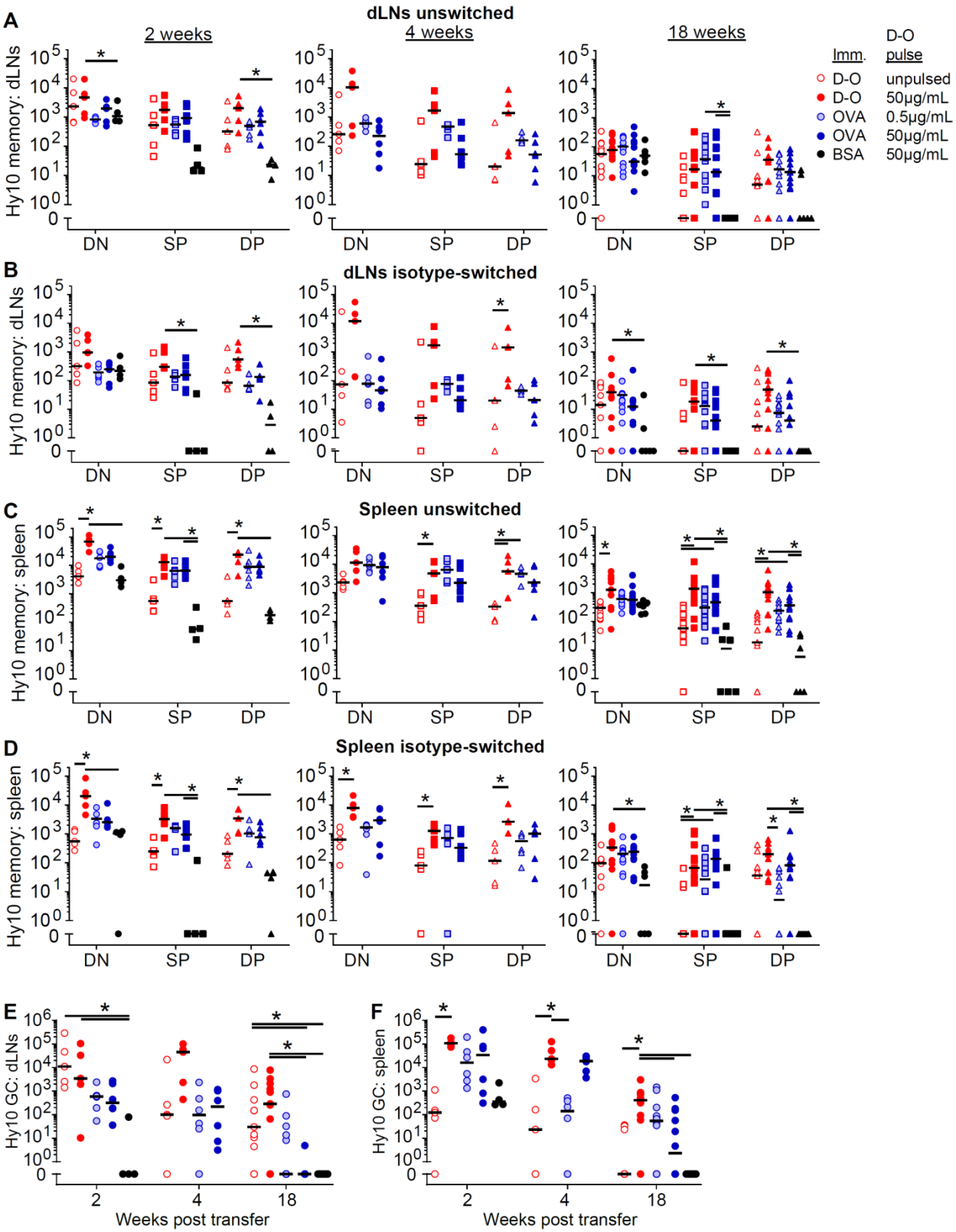

Figure 4.3. Single acquisition of threshold activating amount of Ag enables generation and persistence of memory B cells *in vivo*. Memory (**A–D**) and GC (**E**, **F**) B cell responses of unpulsed (open symbols) and 50 μg/mL (filled symbols) [Legend continues next page]

[Fig. 4.3 continued]
or 0.5 μg/mL (shaded symbols) DEL-OVA pulsed Hy10 B cells in dLNs (**A**, **B**, **E**) and spleens (**C**, **D**, **F**) of OVA (blue symbols), DEL-OVA (red symbols), and BSA (black symbols) immunized recipient mice 2 weeks (left panels), 4 weeks (middle panels) and 18 weeks (right panels) after transfer. DN, SP, and DP subpopulations gated as in **Fig. 4.2A**, **C**, and GCs gated as in **Fig. 4.4A**. All populations shown as total number of cells per dLNs (**A**, **B**, **E**) and spleens (**C**, **D**, **F**). *, $P<0.05$ (Kruskal-Wallis test with Dunn's post-test between all conditions at each timepoint. Differences between groups not annotated by an asterisk did not reach statistical significance.)

Generation of both unswitched and isotype-switched SP and DP memory cells on the other hand, was more similar in dLNs of both DEL-OVA and OVA-immunized mice where T cell help was available, regardless of the amount of Ag B cells initially acquired or their ability to reacquire Ag *in vivo*. At the same time, formation of SP and DP $GL7^{low}$ Hy10 cells was greatly reduced in BSA-immunized control mice (**left panels**, **Fig. 4.1D**, **E and Fig. 4.3A**, **B**). Overall, in OVA or DEL-OVA immunized mice SP and DP B cells made up about 30-50% of total Hy10 $GL7^{low}$ cells, (**left panels**, **Fig. 4.1D**, **E and Fig. 4.3A**, **B**). A similar pattern of memory cell accumulation was observed in spleens of recipient mice, except for an overall less robust response by unpulsed Hy10 cells transferred to DEL-OVA immunized recipient mice (**left panels**, **Fig. 4.1F**, **G and Fig. 4.3C**, **D**). These results suggest that in the presence of T cell help, single transient acquisition of a small amount of Ag by B cells may be sufficient for initial generation of class-switched, SP, and DP subsets of memory B cells.

To determine whether Hy10 memory cells generated in this fashion might have a defect in long-term survival, memory cell persistence was measured at 4 weeks after Hy10 B cell transfer, when resolution of GCs was expected, and 14 weeks later. At 4 weeks a larger population of DP class-switched memory B cells was generated in dLNs and spleens by Hy10 cells that had initially received a large dose of Ag and could reacquire Ag *in vivo* (**middle panels**, **Fig. 4.1E**, **G and Fig. 4.3B**, **D**). These results suggest that over time a larger dose of acquired Ag promotes accumulation of effector-like memory cells. However, in the dLNs of OVA- and DEL-OVA-immunized mice, differences in memory subpopulations among these conditions declined substantially by 18 weeks (**right panels**, **Fig. 4.1D**, **E and Fig. 4.3A**, **B; Fig. 4.2D**). Of note, while DN Hy10 memory cells were detected in BSA immunized recipients at this time, very few SP or DP memory cells persisted in these mice. In the spleen, a larger population

of class-switched DP memory cells was observed in DEL-OVA and OVA immunized mice that received B cells pulsed with a saturating compared to threshold dose of Ag (**right panels**, **Fig. 4.1G and Fig. 4.3D**). Consistent with the similar populations of local memory cells among conditions in the dLNs of DEL-OVA and OVA-immunized mice, we found no substantial difference among these conditions in the early class-switched PC recall response in dLNs, which has been shown to be predominantly mounted by class-switched and DP memory B cells (**Fig. 4.1H and Fig. 4.2E**) (123, 124, 127).

The higher frequency of isotype-switched and DP memory cells observed 4 weeks after transfer of DEL-OVA pulsed Hy10 B cells in DEL-OVA immunized mice may be potentially explained by prolonged residence in GCs by Hy10 B cells that could acquire and reacquire more Ag *in vivo*. However, less prominent differences in the numbers of isotype-switched DP cells observed at 18 weeks suggest that other factors may have contributed to accumulation or persistence of these memory B cells at later times.

We therefore decided to verify duration of Hy10 B cells' persistence in GCs under all conditions. The expectations were first, that the GC response would take place in the Ag-draining LNs but not in the spleen; second, that Hy10 B cells pulsed with a threshold dose of Ag would be more rapidly outcompeted from GCs than B cells pulsed with a larger dose of Ag; third, that Hy10 cells that acquired Ag a single time would be more rapidly outcompeted than B cells that could recurrently acquire Ag *in vivo*; and fourth, that Hy10 GC B cells would exit GCs by 4 weeks following their transfer. GC B cells were defined as $B220^{high}$ $GL7^{high}$ IgD^{low} (**Fig. 4.4A**) and confirmed as $CD38^{low}$ 2 and 18 weeks after transfer (**Fig. 4.4B**). As previously observed, Hy10 cells were recruited into GCs in dLNs following single or recurrent Ag acquisition in DEL-OVA and OVA, but not BSA-immunized mice (**2 weeks**, **Fig. 4.4C**, **D**, **Fig. 4.3E**, and **Fig. 2.1F**, **K**). As expected, 2 weeks after transfer both naïve and Ag-pulsed Hy10 B cells exhibited a trend for more robust GC participation in dLNs of DEL-OVA immunized mice than Ag-pulsed Hy10 B cells in OVA-immunized mice (**2 weeks**, **Fig. 4.4C and Fig. 4.3E**). However, similar GC participation was observed by Hy10 cells that transiently acquired a threshold activating dose of Ag compared to cells pulsed with a saturating dose (**2 weeks**, **Fig. 4.4C and Fig. 4.3E**). Unexpectedly, Hy10 cells that acquired a single saturating or threshold dose of Ag were able to persist for at least 4 weeks in GCs in OVA-immunized mice, while

Hy10 cells transferred into most DEL-OVA immunized mice persisted through the course of the experiment in the dLNs (**Fig. 4.4C and Fig. 4.3E**).

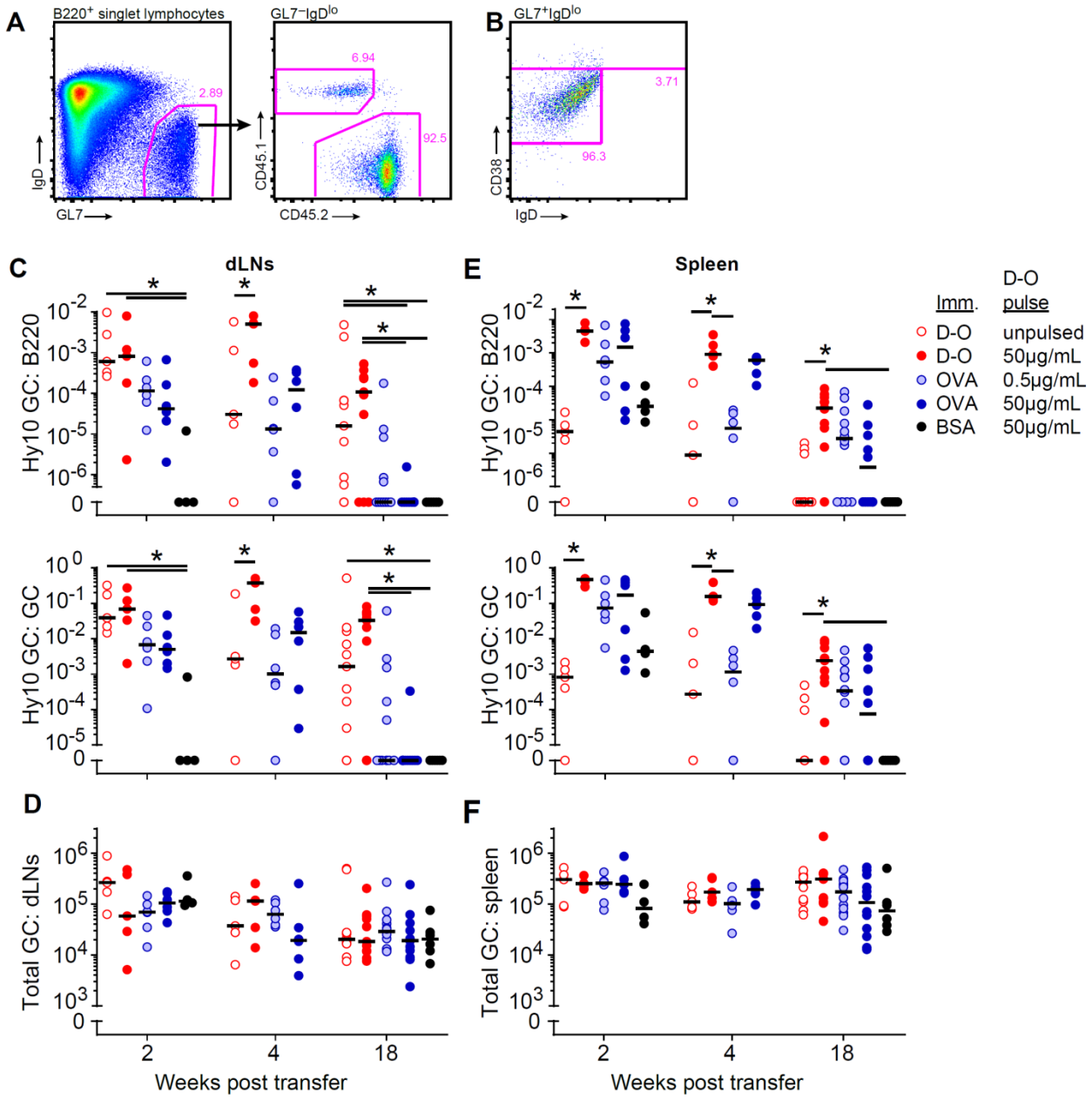

Fig. 4.4. Single acquisition of threshold activating amount of Ag enables GC persistence in spleen. A, Hy10 GC B cell gating strategy. See **Fig. 4.1A** for experimental outline. **B**, CD38 staining of $GL7^{+}IgD^{lo}$ B cells in dLNs 2 weeks after Hy10 transfer to recipient mice. Representative of n=2 independent experiments with 4-6 mice at 2 and 18 weeks after transfer. **C–F**, Hy10 (**C**, **E**) and total (**D**, **F**) GC B cells following transfer of unpulsed (open symbols) and 50 μg/mL (filled symbols) or 0.5 μg/mL (shaded symbols) DEL-OVA pulsed Hy10 B cells in dLNs (**C**, **D**) and spleens (**E**, **F**) of OVA (blue symbols), DEL-OVA (red symbols), or BSA (black symbols) immunized recipient mice [Legend continues next page]

[Fig. 4.4, continued]
at the indicated times post transfer, shown as fraction of total $B220^{+}CD4^{-}CD8^{-}$ singlet lymphocytes (**C**, **E**, upper panels), fraction of total GC B cells (**C**, **E**, lower panels), or total number of GC B cells per tissue (**D**, **F**). For 2 and 4 week timepoints, n=2 independent experiments with 4–6 mice per condition; for 18 weeks, n=4 independent experiments, 6-12 mice per condition. Each symbol represents one mouse, line at median. *, $P<0.05$ (Kruskal-Wallis test with Dunn's post-test between all conditions at each timepoint. Differences between groups not annotated by an asterisk did not reach statistical significance.)

A GC response by Hy10 B cells in the spleen was even less expected. While naïve unpulsed Hy10 B cells mounted little GC response in the spleens compared to dLNs of mice s.c. immunized with DEL-OVA (**Fig. 4.4C–F** and **Fig. 4.3E**, **F**), we observed robust splenic GC participation by Hy10 B cells pulsed with Ag prior to transfer, regardless of whether recipient mice were immunized with DEL-OVA or OVA, and less robust participation in spleens of BSA immunized recipients. At 4 weeks after transfer GC participation by Hy10 B cells pulsed with a threshold dose of Ag decreased ~10–100-fold. However, participation of Hy10 B cells pulsed with a saturating dose of Ag did not substantially decline at this time, regardless of whether they were transferred into OVA or DEL-OVA immunized mice (**4 weeks**, **Fig. 4.4E and Fig. 4.3F**). Finally, by 18 weeks, some Hy10 cells pulsed with either a saturating or threshold dose of Ag and transferred to OVA or DEL-OVA immunized mice still persisted in splenic GCs at similar levels, again regardless of whether they could reacquire Ag *in vivo* (**18 weeks**, **Fig. 4.4E and Fig. 4.3F**). Therefore, we observed that B cells that transiently acquired only a threshold activating dose of Ag not only got recruited into GC responses in non-Ag draining secondary lymphoid organs, but in some recipients also persisted (albeit at low levels) in GCs for at least 4.5 months.

Discussion

In this study we sought to determine whether transiently Ag-pulsed B cells could generate class switched and unswitched $CD80^{-}PDL2^{+}$ and $CD80^{+}$ $PD\text{-}L2^{+}$ functional memory subpopulations, and whether the initial dose of transiently acquired Ag or B cells' ability to reacquire Ag *in vivo* altered their persistence in GCs and generation or survival of functional subpopulations of memory B cells.

We found that even a small dose of transiently acquired Ag was sufficient to enable generation of class-switched and SP and DP memory cells and that acquisition of more Ag or recurrent exposure to Ag did not lead to a significant increase in early (2 week) memory B cell numbers of all tested subtypes. Efficient generation of SP and DP memory cells was dependent on acquisition of T cell help, as Hy10 B cells pulsed with a saturating dose of Ag and transferred to BSA immunized mice generated substantially lower numbers of these cells. However, at one month post transfer the frequency of class-switched DP memory B cells was significantly higher for B cells that were pulsed with a large dose of Ag and could reacquire Ag *in vivo*. The observed difference at this time mirrors the prolonged participation in GCs of Ag-pulsed Hy10 B cells that could reacquire Ag *in vivo* and were pulsed with a saturating dose of Ag. Interestingly, by 4.5 months after transfer, the frequencies of class-switched and unswitched SP and DP memory Hy10 B cells in dLNs decreased approximately 10–15 fold and were not significantly different among non-control conditions. Consistent with that, similar early class-switched PC responses were observed after secondary immunization. While the cumulatively observed results suggest that survival of memory B cells generated after a single transient Ag acquisition by B cells were not defective compared to B cells that recurrently acquired Ag, unambiguous interpretation of these findings is complicated by participation of Hy10 B cells in GCs for substantially longer than expected.

Hy10 B cells were able to persist in GCs in dLNs for 18 weeks or longer when recipient mice had been immunized with cognate Ag. Of note, similarly low-level long term GC persistence has been previously observed following primary s.c. immunization with CFA (123), as well as after viral infection (182) and secondary immunization (124). Such persistence has been attributed to long-term preservation of cognate Ags and their continuous availability to GC B cells. Surprisingly, we observed very efficient recruitment and long-term participation of Ag-pulsed (but not naïve) Hy10 B cells into the GC response in spleens of mice s.c. immunized with either cognate Ag (DEL-OVA) or non-cognate Ag (OVA). While the abundance of Hy10 cells with a GC phenotype dropped over time 100–1000-fold, they persisted in the spleens of some recipients for over 4.5 months. Of note, this was observed even when Hy10 B cells were pulsed with a very low, threshold activating dose of DEL-OVA *ex vivo* and was dependent on the presence of activated cognate helper T cells, as similarly long-term participation was not observed in BSA immunized recipient mice. It is possible that more comparable levels of

memory cells observed 4.5 months after the transfer of Hy10 cells that acquired saturating or threshold activating amounts of Ag could be due to continuous input from these small but persistent splenic GC B cells.

We hypothesize that Ag-pulsed Hy10 cells, which are largely retained in the spleen for ~24-36h (unpublished observations), may be able to recruit cognate Th cells and populate GCs at this site. Because less Ag drains to the spleen, transferred Hy10 cells pulsed *ex vivo* with Ag may be able to outcompete endogenous B cells here. This is in contrast to dLNs, in which Hy10 cells that can reacquire cognate Ag compete more successfully with endogenous B cells than those that cannot reacquire Ag, regardless of whether they were pulsed with Ag prior to transfer. This suggests that competition with endogenous GC B cells is more robust in dLNs and that reacquisition of Ag is necessary for Hy10 B cells to persist in GCs here. Another possible explanation for prolonged GC participation by Hy10 cells in the spleen could be acquisition of cross-specificity to continuously available environmental or endogenous Ags as B cells continuously mutate their BCRs in GCs. Such a phenomenon may explain previously recorded occurrences of GC B cells that have little to no detectable specificity to the immunizing Ag (183).

In this study we also observed that Ag-pulsed Hy10 B cells transferred into BSA immunized control mice expanded, but differentiated into SP and DP memory cells substantially less efficiently than in recipients in which cognate helper T cells had been preactivated. In these mice Ag-pulsed Hy10 B cells also participated at low levels in splenic GCs, but did not persist in them long-term. The observed partial participation of Ag-pulsed Hy10 B cells in the memory and GC response in BSA-immunized mice may be an outcome of a T-independent response of activated B cells in the inflammatory milieu induced by immunization. Alternatively, foreign Ag presentation by Hy10 B cells drives modest activation of endogenous DEL- and OVA-specific Th cells that could, in turn, support Hy10 B cells' participation in some but not all aspects of a T-dependent response.

Overall, in this study we demonstrate that when T cell help is available, transient acquisition of even a threshold activating dose of Ag can enable prolonged participation of B cells in the GC response and generation of various subpopulations of memory B cells, including the class-switched and CD80 PD-L2 based SP and DP memory subsets that quickly differentiate into class switched PCs during recall (123, 124, 127). Therefore, this work suggests that

functional memory cells can be generated following a single Ag acquisition event and may represent a mechanism to promote a wider diversity of Ag-responsive clones to both persistent as well as transiently arising variants of mutating pathogens.

Chapter 5. Individual Contributions of BCR Cross-Linking and T Cell Help in Germinal Center B Cell Selection and Differentiation

Abstract

Generation of high affinity class-switched plasma cells (PCs) and memory B cells is important for protection against many pathogens and requires affinity-based selection of germinal center (GC) B cells. Selection of high affinity GC B cells is thought to proceed according to one or both of two models. The first model suggests that stronger BCR signaling by cells with higher affinity BCRs promotes their survival and selection, whereas the second model proposes that survival signals are derived from Tfh cells and that GC B cells with higher affinity BCRs are selected due to their increased acquisition of Ag for presentation to Tfh cells. While recent studies have provided evidence that competition for T cell help can drive selection of GC B cells, whether Ag acquisition can also support GC B cell selection independently of its role in promoting acquisition of T cell help is not known. We addressed the roles of Ag acquisition and T cell help separately and in combination and found that BCR cross-linking alone is not sufficient to promote GC B cell selection. Preliminary results suggest that BCR cross-linking is not necessary to promote T cell help based selection, but that it may enhance GC B cells' expansion and differentiation into PCs.

Introduction

Selection of high affinity GC B cells is required for differentiation of long lived high affinity PCs that mediate durable protective humoral immunity. Affinity-based selection of GC B cells is thought to be based on competition for Ag and T cell help, but the individual roles of these two factors in promoting selection is not fully understood and is an active area of investigation (184). Several studies have used an elegant technique to examine the effect of increasing T cell help to GC B cells independently of BCR cross-linking by taking advantage of the fact that GC B cells express high levels of DEC205, a cell-surface lectin that delivers Ags it binds to MHCII loading compartments (133). These studies found that administration of DEC-205 antibodies conjugated to T cell Ag (αDEC-Ag) during GC responses increased PC

differentiation and the rate of GC B cell proliferation and hypermutation in a dose-dependent fashion, and that high-affinity GC B cells deficient for DEC-205 were outcompeted by those that expressed the lectin upon αDEC-Ag administration (133, 145, 146). These results suggest that competition for T cell help can drive GC B cell selection. However, recipient mice in these studies were immunized with B cell cognate Ag to initiate GC responses, raising the possibility that integration with signals from the BCR is necessary for αDEC-Ag enhancement of GC responses. Moreover, whether increased BCR cross-linking can promote GC B cell survival or selection alone or in combination with T cell help has not been addressed. Here we examine the contributing roles of Ag and T cell help to GC B cell survival, selection, and effector differentiation.

Materials and Methods

Mice. B6 (C57BL/6) and B6-CD45.1 (Ptprc^{a} Pepc^{b}/BoyJ) mice were purchased from Charles River or the Jackson Laboratory. P14 (B6.Cg-*Tcra*^{tm1Mom} Tg(TcrLCMV)327Sdz) (185, 186) were purchased from Taconic. BCR transgenic (Ig-Tg) Hy10 mice and TCR transgenic OTII mice were generously provided by Jason Cyster (154, 155). Hy10 mice were crossed with B6-CD45.1 mice. All mice were maintained in specific pathogen free environments and protocols were approved by the Institutional Animal Care and Use Committee of the University of Michigan.

Antigen preparation. Duck eggs were locally purchased and lysozyme was purified as previously described (154). Hen egg lysozyme (HEL) and ovalbumin (OVA) were purchased from Sigma. Duck egg lysozyme (DEL) or HEL was conjugated to OVA via glutaraldehyde cross-linking as previously described (154). For production of multimeric DEL (mDEL), purified DEL was conjugated to biotin at a 1:2 molar ratio using biotin-X NHS-ester (Pierce) according to the manufacturer's directions and incubated with purified streptavidin (Thermo Scientific) at a 10:1 molar ratio for 30 minutes on ice followed by removal of unbound DEL-bio by passage through a 30kDa molecular weight cut-off desalting column (Bio-Rad).

For generation of αDEC-OVAp and iso-OVAp, purified DEC-205 and rat IgG2a isotype control antibodies were purchased from Biolegend and partially reduced with 50mM 2-mercaptoethylamine (2-MEA) in PBS, 10mM EDTA for 90 minutes at 37 °C. 2-MEA was removed by passage through 30kDa molecular weight cut-off desalting columns (Bio-Rad), and

half-IgGs were incubated with a 9-fold molar excess of maleimide-substituted OVA peptide 323-339 (Genscript) for 2h at 4 °C. Unbound peptide was removed by passage through 30kDa molecular weight cut-off desalting columns (Bio-Rad) and conjugation was verified by SDS-PAGE.

Adoptive transfer and immunization. Spleens were harvested from male donor OTII mice and pressed through 70μm nylon cell strainers (Falcon) in DMEM (Cellgro) supplemented with 2% FBS (Atlanta Biologicals), 10 mM HEPES, 50IU/mL of penicillin, and 50μg/mL of streptomycin (HyClone). Splenocytes were centrifuged for 7 minutes at 380 rcf, 4°C and resuspended in 0.14M NH_4Cl in 0.017M Tris buffer, pH 7.2 for erythrocyte lysis, washed twice with DMEM supplemented as above, and counted using a Cellometer Auto X4 (Nexcelom). The fraction of $CD19^-$ $CD8^-$ $CD4^+$ $V\beta5^+$ (OTII) splenocytes was determined by flow cytometry, and the indicated number of OTII cells were transferred i.v. to male recipient mice. Ig-Tg B cells were enriched from male or female donor mice by negative selection as previously described (134). For transient exposure to Ag, purified Ig-Tg B cells were incubated with the indicated concentration of DEL-OVA *ex vivo* for 5 minutes at 37°C, washed four times with DMEM supplemented as above, and transferred i.v. to recipient mice. Where indicated, recipient mice were immunized s.c. in the flanks and base of tail with 50μg of the indicated Ag emulsified in complete or incomplete Freund's adjuvant (Sigma), prepared according to the manufacturer's directions. Where indicated, recipient mice were injected s.c.in the base of tail with αDEC-OVAp or iso-OVAp in PBS.

Flow cytometery. Single-cell suspensions from draining lymph nodes were incubated with biotinylated antibodies (Table 1) for 20 minutes on ice, washed twice with 200 μl FACS buffer (2% FBS, 1mM EDTA, 0.1% NaN_3 in PBS), incubated with fluorophore-conjugated antibodies and streptavidin (Table 1) for 20 minutes on ice, washed twice more with 200 μl FACS buffer, and resuspended in FACS buffer for acquisition. For intracellular staining, surface-stained cells were fixed and permeabilized for 20 minutes on ice with BD Cytofix/Cytoperm buffer, washed twice with 200μl BD Perm/Wash buffer, incubated with Alexa 647-conjugated HEL for 20 minutes on ice, followed by two more washes with 200 μl Perm/Wash buffer, and resuspended in FACS buffer for acquisition. Cells were acquired on a FACSCanto and data was analyzed using FlowJo (TreeStar).

Statistics. Statistical tests were performed as indicated using Prism 7 (GraphPad). Differences between groups not annotated by an asterisk did not reach statistical significance. No blinding or randomization was performed for animal experiments, and no animals or samples were excluded from analysis.

Results

To address the individual roles for BCR cross-linking and T cell help in promoting GC B cell proliferation and effector differentiation, three sets of experiments have been undertaken. In the first, we asked whether BCR cross-linking was sufficient to promote GC B cell proliferation or effector differentiation. In the second, we address whether BCR cross-linking within the GC is necessary to enhance T cell help-driven proliferation and effector differentiation, and in the third, we ask whether BCR cross-linking can enhance T cell help-driven proliferation and differentiation in the GC. The second two sets of experiments are ongoing, and data from them are preliminary. In all experiments, purified Hy10 Ig-Tg B cells were pulsed briefly with DEL-OVA *ex vivo*, washed extensively, and transferred into recipient mice in which OTII cells were activated 3d before by immunization with OVA, as previously described. As we have shown before, transient Ag acquisition and T cell help enable Ig-Tg B cells' proliferation and participation in GCs, with recruitment into GCs starting by 4d post transfer (**Fig. 2.2A**). At this point, almost all of the transferred B cells have proliferated, diluting the OVAp acquired from pulsing among daughter cells. This dilution, combined with the lack of cognate Ag in recipient mice means the Ig-Tg B cells recruited into GCs should present lower amounts of OVAp than their endogenous OVA-specific counterparts, and should not receive any stimulation via BCR cross-linking. At this point, recipient mice were injected with reagents that cross-linked Ig-Tg B cells' BCRs, provided additional Ag to present for T cell help, or both.

First, to address whether BCR cross-linking was sufficient to promote GC B cell proliferation or effector differentiation, wild-type or P14 (TCR transgenic for an irrelevant LCMV peptide on TCR$\alpha^{-/-}$ background) recipient mice were re-immunized 4d after transfer with soluble or multivalent DEL (mDEL), which enabled BCR cross-linking but provided no additional antigenic peptides to present to OTII Tfh cells. As positive controls, recipient mice were re-immunized with DEL-OVA to provide both additional BCR cross-linking and peptides to present for T cell help. To establish a baseline of only a single initial transient exposure to Ag,

recipient mice were re-immunized with PBS (**Fig. 5.1A**). As negative controls, DEL-OVA pulsed Ig-Tg B cells were transferred to unimmunized recipient mice with no OTII T cells. Draining iLNs were collected 2 and 4d after re-immunization (6 and 8d after Ig-Tg transfer), and Ig-Tg GC, memory, and PCs were detected by flow cytometry as before (gating strategy, **Fig. 2.2**).

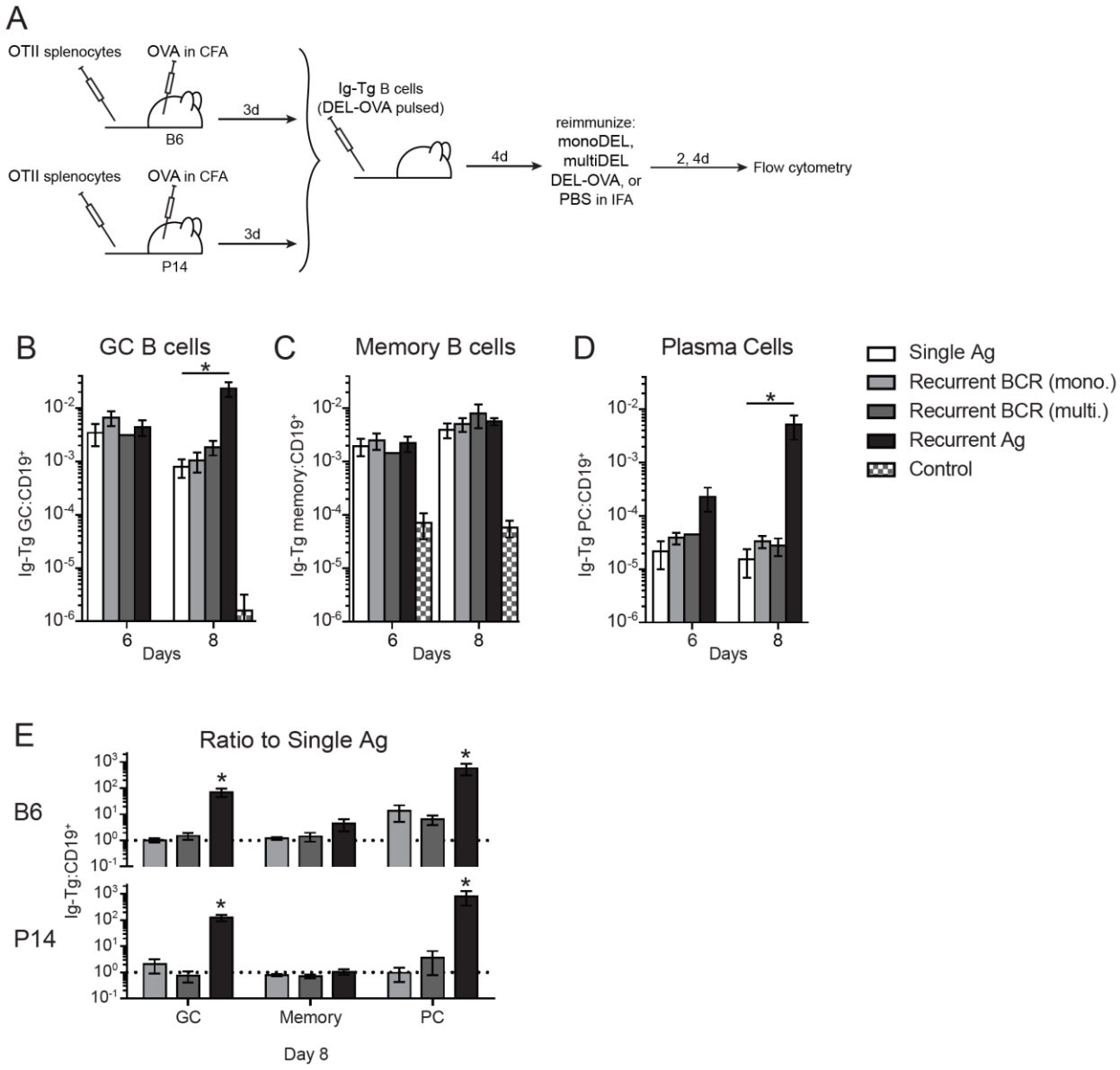

Figure 5.1 BCR cross-linking in the absence of additional T cell help does not promote B cell participation in GC, memory B cell, or PC responses. (**A**) Experimental outline. Purified Hy10 Ig-Tg B cells were pulsed *ex vivo* for 5 min with 50 μg/mL DEL-OVA, washed, and $1x10^6$ were transferred to recipient B6 or P14 mice preinjected with splenocytes containing $5x10^5$ OTII Th cells and s.c preimmunized with OVA in CFA [Legend continues next page]

[Fig. 5.1, continued]
or into unimmunized control mice (not depicted). Four days after Ig-Tg transfer, non-control recipient mice were s.c. re-immunized with sDEL, mDEL, DEL-OVA, or PBS in IFA. (**B–D**) Accumulation of Ig-Tg GC (**B**), memory (**C**), and PCs (**D**) in dLNs from PBS (white bars), sDEL (light grey bars), mDEL (dark grey bars), or DEL-OVA (black bars) re-immunized or control (checked bar) B6 recipient mice at the indicated times after transfer of Ig-Tg cells. See **Fig. 2.2** for gating strategies. (**E**) Ratios of Ig-Tg GC, memory, and PCs formed at d8 by B cells recurrently exposed to Ag to those that acquired Ag only once in B6 (top) and P14 (bottom) recipients. Data from n=3–5 independent experiments, 3–6 mice per condition, shown as mean ± SEM.*p<0.05 (one-way ANOVA).

Compared to DEL-OVA pulsed Ig-Tg cells that received no additional stimulus, recurrent BCR cross-linking alone by either monovalent or multivalent DEL did not promote Ig-Tg cells' participation in GCs, memory B cell differentiation, or PC accumulation (**Fig. 5.1B–E**, **grey bars**). The trend toward a small increase in PC accumulation in response to monovalent or multivalent DEL re-exposure in B6 recipients but not P14 mice (in which the only helper T cells are OTII), suggests that Ig-Tg B cells may have been able to receive help from DEL-specific endogenous T cells in B6 recipients. In contrast, significant increases at day 8 were observed in GC participation and PC accumulation by Ig-Tg B cells that could recurrently acquire both additional BCR cross-linking and T cell help in DEL-OVA re-immunized recipients (**Fig. 5.1 B**, **D**, **E**, **black bars**). Consistent with our previous results that memory B cell differentiation is less sensitive to B cells' ability to reacquire Ag compared to GC participation or PC differentiation, neither additional BCR cross-linking alone or combined with additional T cell help increased memory B cell accumulation at this time point compared to single transient exposure to Ag (**Fig. 5.1C**, **E**). Together these results suggest that BCR signaling is not sufficient to promote GC B cell selection or differentiation into PCs.

Having observed that GC proliferation and PC accumulation were only enhanced under conditions in which additional T cell help was provided, we next asked whether T cell help was sufficient to promote GC B cell proliferation and PC differentiation alone, or whether BCR cross-linking was necessary to enhance these responses. To deliver T cell help to GC B cells without cross-linking the BCR, we targeted T cell Ag to MHCII loading compartments of GC B cells via DEC-205 in similar fashion to previous reports (133, 145, 146). The OVA peptide recognized by OTII T cells was conjugated to DEC205 antibodies (αDEC-OVAp) or to isotype

control antibodies (iso-OVAp). To determine whether T cell help is sufficient to promote GC B cell proliferation and PC differentiation, recipient mice were re-immunized with PBS and injected with 10μg of αDEC-OVAp 4d after transfer of DEL-OVA pulsed Ig-Tg B cells. As positive controls, in which Ig-Tg B cells receive both BCR cross-linking and T cell help, recipient mice were re-immunized with mDEL and injected with αDEC-OVAp. Another set of mDEL re-immunized recipient mice were injected with iso-OVAp to confirm our previous results that BCR signaling alone is insufficient to promote GC B cell proliferation or PC differentiation using this experimental system. As baseline controls in which Ig-Tg B cells encounter single transient exposure to Ag, recipient mice were re-immunized with PBS and injected with iso-OVAp. Finally, as a second set of positive controls and to control for any potential differences in peptide loading between BCR-acquired and DEC205-acquired Ags, recipient mice were re-immunized with DEL-OVA and injected with αDEC-OVAp or iso-OVAp (**Fig. 5.2A**). Draining iLNs were collected 4d after re-immunization and antibody administration (8d after Ig-Tg transfer), and Ig-Tg GC, memory, and PCs were detected by flow cytometry.

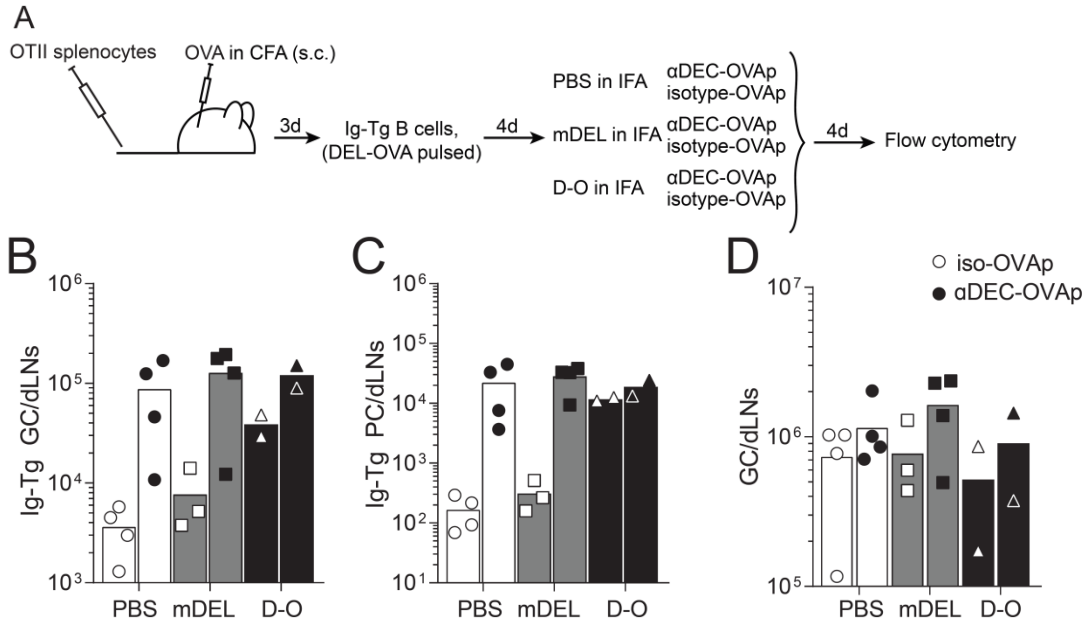

Figure 5.2. BCR cross-linking is not required for T cell help-mediated expansion of GC or PC responses. (**A**) Experimental outline. Ig-Tg B cells were pulsed and transferred into B6 recipient mice with preactivated OTII Th cells as in **Fig. 5.1A**. Four days after Ig-Tg transfer, recipient mice were s.c. re-immunized with mDEL, DEL-OVA, or PBS in IFA and injected with 10μg αDEC-OVAp or iso-OVAp. **B–D**, Ig-Tg GC (**B**), Ig-Tg PC (**C**) and total GC (**D**) accumulation 8d post Ig-Tg transfer in dLNs from PBS (white bars), mDEL (grey bars), or DEL-OVA (black bars) re-immunized mice that received 10μg iso-OVAp (open symbols) or αDEC-OVAp (closed symbols). Data from n=2 experiments. Each symbol represents one mouse.

In preliminary results from 2 experiments, similar GC and PC responses were generated by Ig-Tg B cells in all recipients that received αDEC-OVAp (**Fig. 5.2B**, **C**, **closed symbols**). In PBS and mDEL re-immunized recipients that received αDEC-OVAp, Ig-Tg GC and PC responses were substantially larger compared to those in recipients receiving iso-OVAp (**Fig. 5.2B**, **C**). These results indicate GC and PC responses are enhanced when GC B cells receive additional sources of peptide to present for T cell help, regardless of the presence of BCR cross-linking Ag, and if confirmed, indicate that BCR cross-linking is not required to promote GC B cell proliferation or PC accumulation. Additionally, similar Ig-Tg participation in GC and PC responses was observed in PBS and mDEL re-immunized recipients that received iso-OVAp, consistent with our first set of experiments indicating that BCR cross-linking alone is not sufficient to promote GC B cell proliferation or PC differentiation (**Fig. 5.2B**, **C**, **open symbols**). Taken together, these results are consistent with recent studies emphasizing the role of T cell help in GC B cell selection (70, 71, 133, 145). Of note, the total size of the GC response including endogenous GC B cells was largely consistent across all conditions, regardless of whether recipients were treated with αDEC-OVAp or iso-OVAp (**Fig. 5.2D**). This suggests that the level of OVAp presentation by GC B cells induced by αDEC-OVAp administration is not artificially high. If treatment with αDEC-OVAp increased OVAp presentation beyond the amount presented by endogenous GC B cells acquiring OVA conventionally, an increase in the total GC size compared to iso-OVAp treated recipients would be expected. Rather, the selective increase in Ig-Tg GC and PC responses following αDEC-OVAp administration indicates that this treatment narrows the difference in the amount of OVAp presented by Ig-Tg and endogenous GC B cells. Additionally, the similarity of the GC and PC responses mounted by Ig-Tg B cells in αDEC-OVAp treated recipients following reimmunization with PBS and mDEL compared to DEL-OVA suggests the 10μg dose of αDEC-OVAp used in these experiments delivers a saturating amount antigenic peptide beyond which additional Ag acquisition does not enhance the GC response under these immunization conditions.

This observation raised the question of whether BCR cross-linking, while potentially neither sufficient nor necessary for GC B cell selection, may be able to enhance GC B cell proliferation and/or PC differentiation when T cell help is not saturating. Before addressing this question, the amount of αDEC-OVAp was titrated to establish a dose response curve. To decrease the amount of OVAp initially acquired by Ig-Tg B cells and enhance their sensitivity to

acquisition of supplemental peptide, the concentration of DEL-OVA used for pulsing was reduced to 0.5μg/mL, a threshold activating dose that weakly induces upregulation of CD86 but still enables robust participation in GC and PC responses (**Fig. 2.3**). Furthermore, the number of DEL-OVA pulsed Ig-Tg B cells transferred to recipient mice was reduced to $1x10^5$ to minimize the potential for transfer of Ag among pulsed cells or activation of endogenous DEL-specific Th cells. As before, DEL-OVA pulsed Ig-Tg B cells were transferred to recipient mice in which activated OTII Th cells were present, and recipients were reimmunized with PBS and injected with different amounts of αDEC-OVAp 4d later or with 10μg iso-OVAp as negative controls. Draining iLNs were collected 4d after re-immunization and antibody administration (8d after Ig-Tg transfer), and Ig-Tg GC participation and PC accumulation were analyzed. Based on the results of the titration, 0.5μg αDEC-OVAp was chosen as a dose to promote a small increase in Ig-Tg B cells' GC and PC responses (**Fig. 5.3A**, **B**). To determine whether BCR cross-linking can support GC and PC responses in combination with T cell help, $1x10^5$ Ig-Tg B cells pulsed with 0.5μg/mL DEL-OVA were transferred to recipient mice in which activated OTII Th cells were present, and recipients were reimmunized with PBS, mDEL, or highly multivalent sphDEL and injected with 0.5μg αDEC-OVAp 4d later. Draining LNs were analyzed 3d later (7d post transfer of Ig-Tg B cells) to analyze earlier PC differentiation. The earlier timepoint and addition of highly multivalent sphDEL were used to more closely mirror conditions in a recent study suggesting a role for BCR cross-linking in early stages of GC differentiation into PCs, which used highly multivalent Ag (140). Preliminary results from the first experiment indicated that both the Ig-Tg GC and PC responses were increased following re-immunization with either mDEL or sphDEL compared to PBS in recipients that received 0.5μg αDEC-OVAp (**Fig. 5.3D**, **E**, **filled circles**). Consistent with previous results, reimmunization with mDEL or sphDEL in iso-OVAp treated recipients did not enhance Ig-Tg participation in either the GC or PC responses (**Fig. 5D**, **E**, **open circles**). If confirmed, these results would suggest that BCR cross-linking in GC B cells, while neither sufficient nor necessary for GC B cell selection, may enhance GC and PC responses in combination with T cell help.

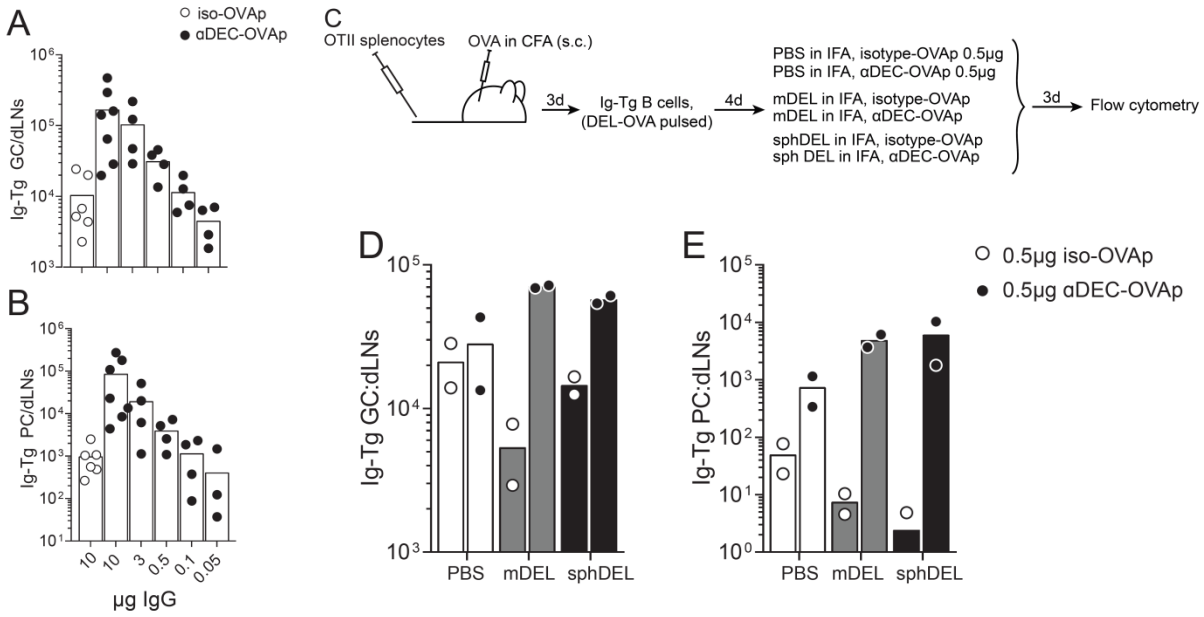

Figure 5.3. BCR cross-linking may enhance GC and PC responses in combination with T cell help. A, **B**, Experimental design as in **Fig. 5.2A**, but with transfer of splenocytes containing $5x10^4$ OTII Th cells and $1x10^5$ purified Ig-Tg B cells pulsed *ex vivo* with 0.5μg/ml DEL-OVA. Ig-Tg GC (**A**) and PC (**B**) accumulation 8d post Ig-Tg transfer in dLNs of PBS re-immunized recipient mice injected with the indicated amount of iso-OVAp (open symbols) or αDEC-OVAp (closed symbols). Data from n=2–3 independent experiments. Each symbol represents one mouse. (**C**) Experimental outline for **D** and **E**. Splenocytes containing $5x10^4$ OTII Th cells were transferred to OVA immunized recipient mice followed by $1x10^5$ Ig-Tg B cells pulsed with 0.5μg/mL DEL-OVA 3d later. Four days after Ig-Tg transfer, recipient mice were s.c. re-immunized with PBS, mDEL, or sphDEL in IFA and injected with 0.5μg αDEC-OVAp or iso-OVAp. (**B**–**D**) Ig-Tg GC participation and PC accumulation 7d post transfer in dLNs from PBS (white bars), mDEL (grey bars), or sphDEL (black bars) re-immunized mice that received αDEC-OVAp (closed symbols) or iso-OVAp (open symbols). Data from n=1 experiment. Each symbol represents one mouse.

Discussion

In ongoing experiments we are attempting to address the individual and combinatorial roles of BCR cross-linking and T cell help in GC B cell selection. While T cell help has been shown to be a potent prosurvival and selection factor for GC B cells, BCR signaling pathways are likely attenuated if not severely restricted in GC B cells (133, 136-138, 145). However, there may be other stimulating signals associated with BCR cross-linking and acquisition of Ag independent of T cell help, such as IL-6 and BAFF produced by FDCs, which are thought to be the primary source of Ag in GCs, or toll-like receptor (TLR) signaling induced by Ags associated with TLR ligands (187-190). Consistent with a role for Ag acquisition in GC B cell selection

beyond promoting T cell help, blocking GC B cells' access to Ag reduced early PC differentiation more than depleting T cells (140). Our experimental design enables us to provide GC B cells at a competitive disadvantage with the means to acquire potential positive selection signals from BCR crosslinking or T cell help, or both in combination.

In the first set of experiments, we tested whether BCR stimulation with monovalent or multivalent Ag could promote GC B cell or PC expansion in the absence of additional T cell help. While reacquisition of Ag containing cognate peptides for Tfh cells increased accumulation of GC B cells and PCs, BCR cross-linking alone did not increase these responses. We next asked whether BCR cross-linking was dispensable for T cell help-mediated GC and PC expansion. To address this question we targeted cognate T cell peptides to MHCII loading compartments via DEC-205, bypassing the BCR. If confirmed, results from these experiments will indicate that BCR cross-linking is not required for T cell help-mediated GC B cell selection. We next asked whether BCR cross-linking can support GC and PC responses in combination with a sub-saturating amount of T cell help, and added a highly multivalent Ag condition to induce more robust BCR cross-linking. Preliminary results from the first experiment indicate that both the Ig-Tg GC and PC responses are increased following re-immunization with either moderately multivalent or highly crosslinking B cell Ag compared to PBS in recipients that received a sub-saturating dose of T cell peptides. If confirmed, the results from this set of experiments will indicate a dominant role for T cell help in mediating GC B cell selection, which may be enhanced by signals mediated by BCR cross-linking.

Chapter 6. Conclusions and Future Directions

While many factors affect recruitment, participation, and effector differentiation of B cells in the T dependent humoral immune response, two of the most critical are acquisition of Ag and T cell help. We sought to address how dynamics of their acquisition affected B cells' initial recruitment into the response, GC participation, and effector differentiation.

Chapter 2 (part 1). Transiently Ag-primed B cells return to a naïve-like state in the absence of T-cell help

Here we addressed the *in vivo* fate of B cells that transiently encounter Ag in the absence of T cell help. We found that transient acquisition of moderately multivalent Ag does not induce B cell death or anergy. Rather B cells downregulate activation markers and relocalize from the T/B border back into follicles, survive similarly to naïve B cells for at least a week, and can be recruited into a subsequent immune response following reacquisition of Ag and T cell help. In contrast, B cells that transiently acquire highly multivalent Ag or continuously acquire moderately multivalent Ag in the absence of T cell help demonstrate a defect in survival, consistent with previous reports of the negative impacts on B cell survival of very strong BCR signaling and chronic exposure to Ag in the absence of T cell help (113, 153).

Tolerance can also be induced in B cells that continually acquire low-avidity Ag in the absence of secondary activating signals through induction of anergy, a state characterized by exclusion from follicles, functional unresponsiveness to stimulation with Ag and T cell help, and a shortened half-life of approximately 3 days (105, 106, 113). The reduced half-life of anergic B cells is due to an increased dependence on the prosurvival factor BAFF to offset BCR signaling-mediated upregulation of the proapototic protein Bim (109, 110). Bim is transiently upregulated in Ag-pulsed cells, and may induce a similar, though less severe increase in BAFF dependence that could be met by physiological levels of BAFF, and explain the difference in survival of Ag-pulsed cells observed *in vivo* and *ex vivo* (116) and (**Fig. 2.4**). Experiments are ongoing in the lab to address whether BAFF can rescue *ex vivo* survival of Ag-pulsed B cells.

Anergic B cells removed from continuous exposure to cognate Ag rapidly restore many aspects of normal BCR signaling, and can generate PC responses upon restimulation with Ag

and T cell help (105, 191, 192). However, their responses are less robust than those generated by naïve B cells, suggesting that some aspects of the anergic state are more durable (105). We demonstrated that B cells that had been transiently activated and returned to quiescence generated GC and PC responses with similar efficiency as naïve B cells upon reimmunization with a non-limiting amount of Ag and in the presence of abundant T cell help (**Fig 2.9**). It is possible that the abundance of Ag and T cell help in these experiments masked potentially altered sensitivities to these factors in B cells that have returned to quiescence. Increased or decreased sensitivities could be revealed by assessment of upregulation of activation markers such as CD69 or CD86, or extent of proliferation, GC participation, or effector differentiation compared to naïve B cells in response to decreased amounts of Ag or limiting T cell help.

One potential caveat to our studies is the use of transgenic B cells, which may have different signaling or survival properties and participate in humoral immune responses differently than non-transgenic polyclonal B cells. Transient stimulation of wild type B cells could be performed with IgM-specific antibodies as was done previously in a study of the BCR signaling response to transient BCR stimulation (116). Polyclonal cells specific for PE or APC could be also be isolated using magnetic enrichment as previously described; this purification strategy would also serve to provide the B cells with a single dose of Ag (123). However the affinities of the Ag specific cells isolated using this strategy would vary, potentially complicating interpretation of the results.

Chapter 2 (part 2). Transiently Ag-primed B cells are recruited into T-dependent humoral immune responses

Here we addressed whether transient acquisition of Ag can enable B cell participation in the T-dependent humoral response when cognate T cell help is readily available. We found that B cells transiently primed with moderately multivalent high- or medium-affinity Ag proliferated, were recruited into GCs, and differentiated into memory B cells and PCs in response to T cell help. These results indicate that continuous or recurrent exposure to Ag is not required for B cells' full participation in T-dependent humoral responses *in vivo*. While transient acquisition of a broad range of Ag amounts enabled efficient recruitment of B cells into the response and similar generation of memory cells, PC accumulation and, to a slightly lesser extent, GC participation were more sensitive to the initially acquired amount of Ag, consistent with increased competition for T cell help during differentiation and expansion of these cells (54,

133). Timely availability of T cell help was required for transiently Ag-pulsed cells' recruitment into the response; their ability to productively acquire T cell help and proliferate declined by half within 24 hours of transient Ag acquisition and was completely abolished between 36 and 48 hours, in parallel with their downregulation of activation markers.

B cells' access to large particulate Ag may be restricted during the initial stages of the immune response, favoring a transient mode of Ag acquisition, which we have demonstrated favors B cell survival in the absence of T cell help. This may represent a mechanism by which Ag-specific B cells can preserved from induction of anergy or apoptosis induced by continuous BCR stimulation before cognate helper T cells have had time to expand. This hypothesis is consistent with the observation that soluble Ags elicit less robust humoral immune responses than larger and more particulate Ags (83, 163). Future studies could compare early survival of Ag-specific B cells following injection of soluble and particulate Ags and the impact this has on the responses elicited by each.

During the later stages of an immune response, when T cell help is readily available, Ag has likely redistributed into follicles and is more available for acquisition by B cells. A potential scenario in which T cell help could be readily available but availability of Ag could be limited, favoring a transient mode of acquisition by B cells, is during infection with a mutating pathogen, and a B cell recognizes a rare mutant, but not the parent strain. Our results suggest that under such a scenario, the mutant-specific B cell would be able to acquire T cell help, proliferate, efficiently differentiate into memory cells and be recruited into GCs, and less efficiently, produce PBs and compete within the GCs. Experimentally, B cells' ability to be recruited into the humoral immune response under such conditions could be tested by transiently pulsing Ig-Tg B cells with a lysozyme-coated virus-like particle and transferring them to mice infected with the corresponding virus.

Chapter 3. Ag acquisition enables newly arriving B cells to enter ongoing immunization-induced GCs

Here we determined that the ability of naïve Ag-specific B cells to acquire Ag is one of the factors limiting their recruitment into ongoing immunization-induced GCs. We found that preloading B cells with Ag enabled their recruitment and participation in histologically-defined GCs throughout the initiation, peak, and contraction phases of the GC response, regardless of their ability to reacquire Ag *in vivo*. Despite efficient recruitment during all phases of the GC

response, B cells preloaded with Ag were limited in their expansion and generation of class-switched memory B cells if transferred after the peak of the GC response; this corresponded with a decreased fraction of Tfh cells that produced IL-21 and an increased ratio of Tfr cells to Tfh cells within the GC. In contrast to B cells preloaded with Ag, naïve Ag-specific B cells were severely limited in their ability to be recruited into GCs after the peak of the GC response; this corresponded with their inability to upregulate the early activation marker CD69, and combined with Ag-pulsed B cells' efficient GC recruitment indicated that availability of Ag for acquisition by naïve B cells is limited during later phases of the GC response.

An alternative possibility is that accumulation of Ag in immune complexes at later stages of the response could induce inhibitory Fc signaling in naïve B cells, limiting their activation rather than availability of Ag per se. If this is the case, Ags containing PAMPs may be able to balance the inhibitory Fc signal through pattern recognition receptor signaling.

Chapter 4. Transiently Antigen Primed B Cells Can Generate Multiple Subsets of Memory Cells.

Here we address how dynamics of Ag acquisition alter memory B cell differentiation and generation of different memory B cell subsets. We found that when T cell help is available, transient acquisition of even a threshold activating dose of Ag can enable generation of various subpopulations of memory B cells, including the class-switched and CD80 PD-L2 based SP and DP memory subsets that quickly differentiate into class switched PCs during recall. These results are consistent with our previous findings that in comparison to PC and GC B cell expansion, generation of memory B cells was less dependent on B cells' ability to reacquire Ag or on the initial dose of transiently acquired Ag. Surprisingly, we also found that transient acquisition of Ag enabled prolonged, low-level participation of B cells in the GC response.

The mechanisms enabling persistent GC participation by B cells that transiently acquired Ag are unclear. One possibility is that in the spleens of recipient mice, competition with endogenous B cells is less robust in spleens of recipient mice, as less Ag drains here than the iLNs following s.c. immunization. Early GC participation of Ag-pulsed cells may therefore be more robust. Whether cognate Ag is maintained and sustains GCs or if the transferred Ig-Tg B cells acquire specificity to endogenous or environmental Ags is not known. To help determine between these possibilities, the Ag-specificity of Ig-Tg B cells in persistent GCs should be determined.

As these persistent GCs could represent a source of continual input of memory B cells, differences in the longevity of memory B cells generated in response to transient and recurrent Ag acquisition could be masked. Sorting and adoptive transfer of memory B cells into naïve recipients would allow these potential differences to be assessed.

Chapter 5. Individual Contributions of BCR Cross-Linking and T Cell Help in Germinal Center B Cell Selection and Differentiation

Here we address the individual and combinatorial roles of BCR cross-linking and T cell help in GC B cell selection. We first asked whether BCR cross-linking alone, without providing additional peptides to present for T cell help could act as a positive selection signal for GC B cells, and found that it was insufficient to promote GC B cell expansion or PC accumulation.

We next asked whether T cell help was sufficient to promote GC B cell proliferation and PC differentiation alone, or whether BCR cross-linking was necessary to enhance these responses. In preliminary results we find that increasing the amount of cognate T cell peptides independently of BCR crosslinking promoted GC B cell expansion and PC accumulation. If confirmed, results from these experiments will indicate that BCR cross-linking is not required for T cell help-mediated GC B cell selection. We next asked whether BCR cross-linking can enhance GC and PC responses in combination with a sub-saturating amount of T cell help, and found in very preliminary results from the first experiment that in combination with a sub-saturating amount of T cell peptide, BCR cross-linking can enhance GC and PC responses.

One caveat to these preliminary results suggesting BCR cross-linking can enhance GC B cell selection is the potential for Ig-Tg B cells' acquisition of T cell help from DEL-specific endogenous T cells in the B6 recipients used in this experiment, as appeared to be the case in the first set of experiments. This possibility is mitigated to some extent by the reduced dose of DEL-OVA used to pulse the Ig-Tg B cells and the lower number of Ig-Tg B cells transferred, which both reduce the overall amount of DEL peptide presented by activated B cells, as well as the earlier timepoint (day 7 instead of 8), which reduces the amount of time DEL-specific Tfh cells would have to differentiate. Nonetheless, this possibility will be more rigorously controlled for in future experiments, which will use $SAP^{-/-}$ recipient mice, in which endogenous T cells are incapable of differentiating into Tfh cells. Similar to P14 recipients in the first set of experiments, transferred OTII T cells in these experiments will be will be the only source of Tfh cells.

To help determine whether BCR signaling contributes to GC B cell selection, activation of the BCR signaling pathway will also be assessed in future experiments to help determine if BCR signaling is induced in GC B cells by mDEL or sphDEL. This may help determine whether BCR signaling per se can promote GC B cell selection as opposed to other non-T cell help mediated effects of Ag acquisition, such as increased exposure to IL-6 or BAFF from FDCs.

Most of the studies of mechanisms of GC B cell selection have analyzed selection at the peak of the GC response, including ours. Future studies should address how the mechanisms of selection differ during the later stages of the GC response and in persistent GCs; the profile of Tfh cells change as the response proceeds, and therefore the impact of T cell help may also change (73) and (**Fig. 3.1**). Additionally, other signals such as BCR signaling, FDC-derived stimuli, or pattern recognition receptor signaling may have different roles in GC selection during later stages of the GC response.

Altogether, we found that transient Ag acquisition enables efficient recruitment into the T-dependent humoral immune response, including participation in GCs and differentiation of memory B cells. We demonstrate that reacquisition of Ag is dispensable for recruitment into all phases of the T-dependent response, including GC responses and generation of class-switched PCs and effector-like memory B cells. B cells face increased requirements for reacquisition of Ag for GC selection and robust PC differentiation, but efficient memory cell generation after transient Ag acquisition may represent a necessary balance the immune system must strike between the necessity of selecting the highest affinity Abs to clear ongoing infections and recruiting a broader diversity of clones that could help protect against related strains of pathogens.

APPENDIX

Table 1. List of Antibodies

Target	Application	Species	Fluorophore(s)	Clone	Dilution(s)	Source
B220	FC	Rat	FITC, PerCP-Cy5.5, PE-Cy7, V500	RA3-6B2	50, 100, 100, 50	BD Pharm.
Bcl6	IF	Mouse	A647	K112-91	25	BD Pharm.
CD3	IF	Arm. Ham.	PE-Texas Red	145-2C11	100	BD Biosci.
CD4	FC	Rat	APC-Cy7	RM4-5	100	BioLegend
CD4	FC	Rat	V500	RM4-5	400	BD Biosci.
CD8	FC	Rat	APC-Cy7	53-6.7	100	eBioscience
CD8	FC	Rat	V500	53-6.7	50	BD Biosci.
CD11c	MACS	Arm. Ham.	Biotin	N418	100	BioLegend
CD19	IF	Rat	Biotin	6D5	100	BioLegend
CD19	FC, IF	Rat	PE	1D3	100	BD Pharm.
CD25	FC	Rat	BV421	PC61	50	BioLegend
CD38	FC	Rat	PerCP-eFluor 710	90	200	eBioscience
CD43	MACS	Rat	Biotin	S7	100	BD Biosci.
CD45.1	IF, FC	Mouse	PE, Pacific Blue, Alexa 647, Alexa 700	A20	100, 100, 100, 50	BioLegend
CD45.2	FC	Mouse	Pacific Blue, Alexa 700, PerCP-Cy5.5	104	50	BioLegend
CD69	FC	Arm. Ham.	PE	H1.2F3	25	BD Pharm.
CD80	FC	Arm. Ham.	PerCP-Cy5.5	16-10A1	100	BioLegend
CD86	FC	Rat	Biotin, Alexa 647	GL-1	400, 100	BioLegend
CD95	FC	Arm. Ham.	PE-Cy7	Jo2	100	BD Pharm.
CD138	FC	Rat	Biotin	281-2	100	BD Pharm.
CD197	FC	Rat	Biotin	4B12	10	BioLegend
CD273	FC	Rat	APC	TY25	100	BioLegend
CD279	FC	Rat	PE-Cy7	RMP1-30	100	BioLegend
CXCR5	FC	Rat	Biotin	2G8	25	BD Biosci.
FoxP3	FC	Rat	APC	FJK-16s	25	eBioscience
GL-7	FC	Rat	Biotin, eFluor 450, eFluor 660	GL-7	100	eBioscience
I-A/I-E	FC	Rat	PerCP-Cy5.5	M5/114.15.2	200	BioLegend
I-A^b/Ea	FC	Mouse	Biotin	Y-ae	100	eBioscience
IgD	FC	Rat	PerCP-Cy5.5, APC-Cy7	11-26c.2a	100	BioLegend
IgD	IF	Rat	FITC	11-26c.2a	100	BioLegend

Target	Application	Species	Fluorophore(s)	Clone	Dilution(s)	Source
IgH+L	FC	Goat	Alexa 647	Polyclonal	100	S. Biotech
IgG_1	FC	Rat	Biotin	A85-1	100	BD Pharm.
IgG_1[a]	ELISA	Mouse	Biotin	10.9	1000	BD Pharm.
IgM	FC	Goat	FITC	Polyclonal	100	S. Biotech
IgM[a]	FC	Mouse	PE	DS-1	200	BD Pharm.
IgM[a]	ELISA	Mouse	Biotin	DS-1	4000	BD Pharm.
IgM[b]	FC	Mouse	PE	AF6-78	200	BD Pharm.
IFN-γ	FC	Rat	A700	XMG1.2	200	BioLegend
IL-21	FC	Rat	PE	FFA21	200	eBioscience
Vβ5	FC	Mouse	PE	MR9-4	200	BD Pharm.
S.avidin	FC	n/a	Qdot 605	n/a	200	Life Technol.
S.avidin	IF	n/a	Dylight 488	n/a	200	BioLegend
S.avidin	IF	n/a	Alexa 647	n/a	200	Life Tech.
Legend						
FC – Flow Cytometry						
IF – Immunofluorescence						
MACS – AutoMACS purification						
ELISA – Enzyme-Linked Immunosorbent Assay						

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