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Article

Initial Clonal Expansion of Germinal Center B Cells Takes Place at the Perimeter of Follicles

Francis Coffey,[1,2] Boris Alabyev,[1,2,3] and Tim Manser[1,*]
[1]Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[2]These authors contributed equally to this work
[3]Present address: Nanogen Inc., Bothell, WA 98021, USA
*Correspondence: manser@mail.jci.tju.edu
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SUMMARY

Current models of the germinal center (GC) response propose that after stimulation at the edges of T cell zones, pre-GC B cells directly migrate to the center of follicles and proliferate to form GCs. We followed the interrelationship of proliferation, differentiation, and microenvironmental locale in populations of pre-GC B cells responding to antigen. In contrast to the predictions of current models, after accumulation at the T-B interface, these cells appeared at the perimeter of follicles adjacent to the marginal zone. There, they rapidly proliferated for several days but underwent no V gene hypermutation and little heavy-chain class switching. Their chemokine receptor expression pattern indicated that these cells were sessile, yet they had begun to acquire many phenotypic characteristics of GC B cells. The expanded clones were subsequently observed in the center of follicles, suggesting that GCs are created by coalescence of B cells from this follicular perimeter response.

INTRODUCTION

During T cell dependent (TD) immune responses, B cells undergo proliferation, differentiation, and selection in the microenvironment of the germinal center (GC), thereby leading to the formation of the memory B cell compartment. According to many models, the GC response takes place in a series of discrete stages (Kelsoe and Zheng, 1993; MacLennan, 1994; Liu and Arpin, 1997; Camacho et al., 1998). After their antigen receptors (BCRs) engage antigen, follicular B cells migrate to near the border of T zones and undergo cognate interaction with helper T cells. Subsequently, pre-GC B cells travel to the center of the follicle and rapidly proliferate in association with follicular dendritic cells (FDCs), resulting in formation of the GC proper. It has been suggested that during this early phase of clonal expansion, changes in the structure and function of BCRs do not occur, and this results in the generation of a sufficient pool of precursors to sustain the somatic-hypermutation-selection and differentiation processes that ensue (Jacob et al., 1991).

Histologic and flow cytometric GC "time course" studies have provided support for this idea (Berek et al., 1991; Jacob et al., 1991; Jacob and Kelsoe, 1992; McHeyzer-Williams et al., 1993; Camacho et al., 1998; Vora et al., 1999), but their limited resolution has not allowed a distinction between initial B cell clonal expansion in and recruitment to the GC. Moreover, recent multiphoton imaging studies of ongoing GC reactions in lymph nodes have revealed a far greater degree of plasticity of GC B cell behavior than predicted by previous models of this response (Allen et al., 2007; Schwickert et al., 2007; Hauser et al., 2007). In particular, these analyses demonstrated extensive migration of individual GC B cells within, around, into, and out of the GC proper.

Given the above considerations, we performed a detailed analysis of the interrelationship of proliferation, differentiation, and microenvironmental locale in homogenous populations of pre-GC B cells during the early stages of TD immune responses. These studies revealed a previously unappreciated stage of the GC B cell developmental pathway, in which pre-GC B cells undergo rapid clonal expansion at the perimeter of follicles without induction of somatic hypermutation, heavy-chain class switching, or expression of genes necessary for homing to the center of follicles. Our findings necessitate revision of previous models for the GC response.

RESULTS

V_H Gene-Targeted Transgenic Mice

We utilized a previously described line of *Igh* locus V_H gene-targeted transgenic mice termed HKI65 for our studies (Heltemes-Harris et al., 2004). B cells in these mice express a high frequency of BCRs that bind the hapten arsonate (Ars) and are encoded by a single V gene segment combination and thus are termed "canonical." Such targeted V_H loci efficiently undergo somatic hypermutation (SHM) and class switch recombination (CSR) (Taki et al., 1995; Shih et al., 2002). For most experiments, HKI65 $Igh^{+/-}$ mice were mated to a previously described line of immunoglobulin (Ig) light (L) chain transgenic mice expressing the canonical Vk10A-Jk1 L chain gene (Liu et al., 2007), creating double-transgenic mice we term HKI65-Vk10. Greater than 95% of B cells in these mice express canonical BCRs that are readily detected in histological and flow cytometric analyses with the anticlonotypic mAbs E4 (Notidis et al., 2002; Heltemes-Harris et al., 2004) or 5Ci (Wysocki and Sato, 1981; B.A., unpublished data).

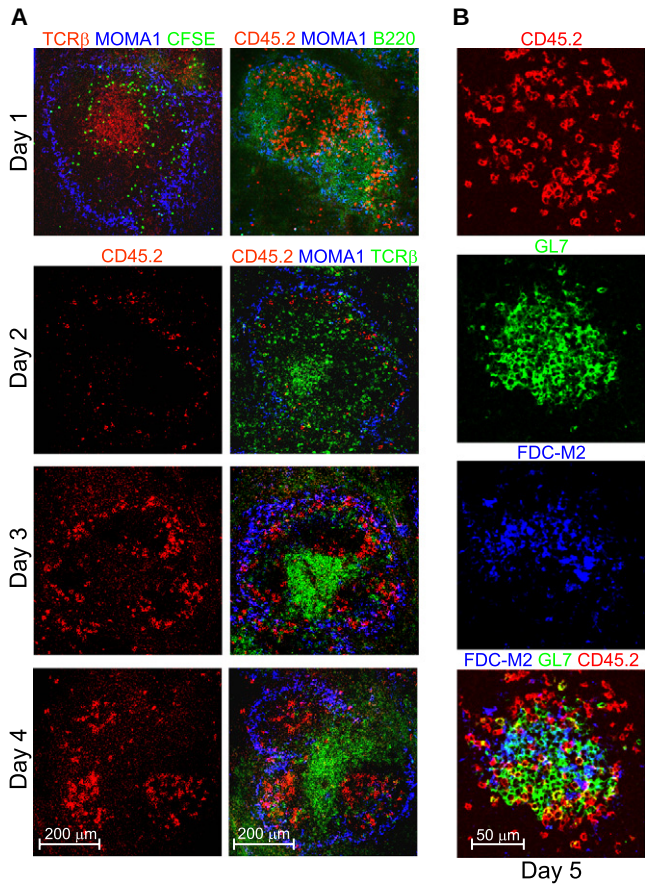

Figure 1. Microenvironmental Locale of HKI65-Vk10 Donor B Cells during the First Five Days of the Immune Response in Ars-KLH Preimmunized Mice

Recipient mice were primed with 100 μg of Ars-KLH in alum i.p. and injected with HKI65-Vk10 B cells 1 week later; this was followed by a single injection of Ars-KLH in PBS as described in Experimental Procedures. Mice were sacrificed at different time points and spleen sections were analyzed.

(A) Day 1, left panel illustrates the localization of CFSE-labeled donor B cells (10^7 injected per recipient mouse), which appear green, on the first day after transfer. Day 1, right panel shows donor B cells (10^7 injected per recipient mouse) expressing the CD45.2 allotypic marker (red) concentrated at the interface of B (green) and T (unstained) cell zones. The remaining panels illustrate the location of CD45.2$^+$ donor B cells (red, 2×10^4 injected per recipient mouse) at days 2–4 after immunization (adjacent sections next to each other). We used MOMA1 staining (blue) to identify metallophiles in the MZ, delineating the follicular border. T cells appear green. The data shown are representative of those obtained from at least three mice from each time point.

(B) Representative images of a germinal center at day 5 after transfer. Donor B cells (2×10^4 injected per recipient mouse) stained with anti-CD45.2 appear red, GL7-positive cells appear green, and FDC-M2 staining appears blue. The scale is shown below each column of panels.

HKI65-Vk10 B Cells Are Located at the Perimeter of Follicles during the Early Anti-Ars Response

Splenic B cells enriched from HKI65-Vk10 mice were injected into immunosufficient, unirradiated, nontransgenic, syngeneic recipients that had or had not been immunized intraperitoneally (i.p.) with Ars-keyhole limpet hemocyanin (Ars-KLH) in alum 1 week earlier. All recipient mice used in these studies were incapable of expressing canonical BCRs because of absence of the requisite V_H gene segment. This preimmunization approach was initially taken because we anticipated that it would result in prior development of a KLH-specific helper T cell compartment sufficient to induce synchronous activation of many Ars-specific donor B cells. Such an approach would allow effective evaluation of various stages of their subsequent response to antigen in vivo. In some experiments, donor cells were labeled with CFSE to allow monitoring of cell division (Lyons and Parish, 1994). Also, in most studies, recipient mice were strain C57BL/6.CD45.1 (B6.CD45.1) and as such permitted distinction of donor and host B cells with the CD45.1-CD45.2 allotype system, irrespective of the structure and expression of their BCRs.

One day after transfer, donor B cells were mainly found by histological analyses to be scattered throughout follicles in unimmunized chimeric mice, a distribution that persisted for 10 days. Flow cytometric analysis at all time points after transfer did not reveal proliferation or expression of the GC activation marker GL7 by donor B cells in these mice (data not shown). B cells transferred to immunized mice were also observed in follicles on day 1. However, a major subset of these cells was concentrated at the interface of follicles and the periarteriolar lymphoid sheath (PALS) T cell zones (Figure 1A, top row), where initial cognate interaction of antigen-specific B cells and T cells most probably takes place (Jacob et al., 1991; Liu et al., 1991; Jacob and Kelsoe, 1992; Martin and Goodnow, 2002; Pape et al., 2003).

By day 2, staining with anti-CD45.2 (Figure 1A) and 5Ci (data not shown) revealed small numbers of donor B cells located at the perimeter of follicles, near the border with the marginal zone (MZ) (defined by staining of metallophillic MZ cells with the mAb MOMA1). Most donor cells in this locale were distal to the PALS (Figure 1A, day 2). By day 3, donor B cells were numerous in this region (Figure 1A, day 3). At day 4, donor B cells were abundant in the center of follicles (Figure 1A, day 4). By day 5, most of these cells were located in GCs, as defined by staining with GL7 for GC B cells and FDC-M2 for FDCs (Figure 1B). These results suggested that canonical HKI65-Vk10 B cells were undergoing clonal expansion at the perimeter of follicles prior to entering the GC reaction.

HKI65-Vk10 B Cells Undergo Rapid Proliferation at the Perimeter of Follicles Prior to Entering GCs

To test this idea, we performed histological studies by using a mAb specific for the nuclear proliferation antigen Ki67 and flow cytometric analyses for dilution of CFSE in donor B cells. Figure 2A (day 1, top row) illustrates that only a small percentage of donor B cells present in and around the T cell zone at day 1 were Ki67$^+$. In marked contrast, by days 2 and 3, the majority of these B cells, now located at the perimeter of follicles, were Ki67$^+$ (Figure 2A, day 2 and day 3). The results of quantification of such data are presented in Figure 2B. As expected from previous studies (Camacho et al., 1998; Rahman et al., 2003), by day 4, nearly all donor B cells, now located in GCs in the center of follicles, were Ki67$^+$ (Figure 2A, day 4). In addition, BrdU-pulse-labeling studies showed that anti-clonotype$^+$ B cells in the S phase were numerous in the locale of the perimeter of follicles in the day 2 to 4 time frame (data not shown). These data showed that HKI65-Vk10 B cells are actively proliferating while they are located at the follicular perimeter.

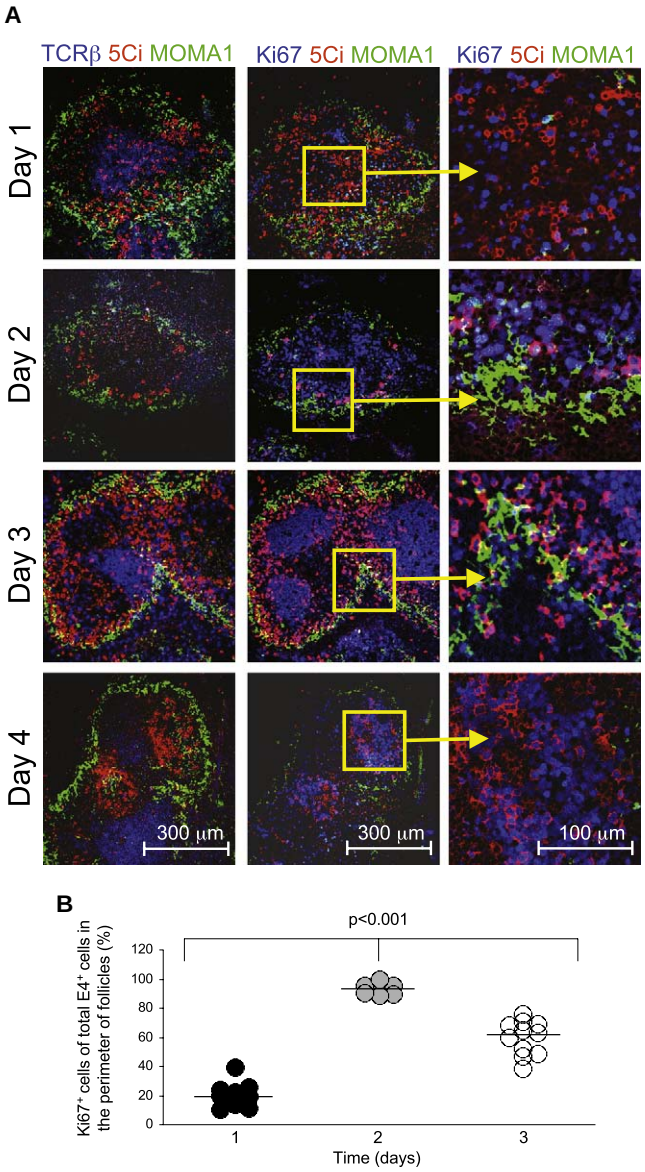

Figure 2. Proliferation of HKI65-Vk10 Donor B Cells at the Perimeter of Follicles in Preimmunized Mice

Chimeric HKI65-Vk10 mice were created and immunized as described in the legend to Figure 1.

(A) Panels in the left column illustrate the location of donor 5Ci$^+$ B cells (red), T cells (blue), and MZ metallophiles (green) at different time points during the course of the Ars-KLH response. Panels in the center column show the result of staining of adjacent sections for the Ki67 nuclear proliferation antigen (blue), donor B cells (red), and MZ metallophiles (green). The right column of images shows magnified regions of areas delineated by yellow rectangles in the center panels. The scale is shown in the lower panel of each column. Data were obtained from mice injected with the following numbers of donor B cells: day 1, 10^7; day 3, 10^6; days 2 and 4, 2×10^5. The data shown are representative of those obtained from at least three mice from each time point.

(B) Quantitative analysis of E4$^+$ B cell proliferation at the perimeter of follicles on the indicated days. Data obtained represent the percentage of canonical HKI65-Vk10 E4$^+$ B cells that were also Ki67$^+$ counted within 15 cell diameters from the band of MOMA1$^+$ cells that delineate the MZ-follicle border. Each circle represents data from an individual field along this border. Data were obtained from three mice for days 1 and 3 and from two mice for day 2. The p values were calculated with a two-tailed Student's t test.

Localization and Proliferation of Pre-GC B Cells at Follicular Perimeters without Preimmunization

Introduction of naive donor B cells into mice with ongoing immune responses and GC reactions clearly does not directly mimic conditions at the onset of a conventional immune response. As such, we conducted an extensive series of studies in which naive HKI65-Vk10 splenic B cells were injected into nonirradiated, syngeneic recipients; this was followed by i.p. immunization of the resulting chimeric mice with Ars-KLH in alum 12 hr later. Control studies revealed few, if any, GCs in the spleens of naive recipient mice prior to cell transfer and immunization (data not shown). Figure 3 illustrates that results entirely analogous to those obtained with the preimmunization protocol were obtained with this postimmunization approach, although donor B cell localization to and proliferation in the follicular perimeter and subsequent donor B cell entry into GCs were delayed by 1–2 days as compared to the preimmunization situation, and the response of these donor cells appeared less synchronous.

We next combined CFSE labeling of donor B cells with histological and flow cytometry analyses to extend the resolution of these postimmunization protocol studies. At day 2 after immunization, donor B cells were predominantly located at the interface of the follicle and the PALS and had not undergone detectable levels of proliferation (data not shown). Figure 4A illustrates that at day 3 after immunization, donor B cells were located both at the interface of the follicles and PALS and in the follicular perimeter. Flow cytometric measurement of CFSE dilution and other markers revealed that the majority of donor B cell had not undergone proliferation at this time point (Figure 4C, upper-left panel). By day 4, however, most donor B cells were located at the follicular perimeter and many were Ki67$^+$ (Figure 4B). Figure 4B, lower left, illustrates that all of the donor B cells located in this region lacked CFSE fluorescence, most probably because of the fact that active proliferation had diluted their levels of CFSE below the detection limit of fluorescence microscopy. Flow cytometry demonstrated that a major fraction of these cells had divided two to four times (Figure 4C, lower panels). Figure 4C also illustrates that while proliferating, donor B cells at days 3 and 4 had begun to acquire the GL7 activation marker and expressed progressively lower amounts of surface IgD. They had not yet become PNA$^+$ and did not express detectable surface IgG.

Analogous results were obtained with the preimmunization protocol (Figure S1 available online). However, in these studies nearly all donor B cells were recruited into the proliferative response, and this response was accelerated by several days. Also, proliferating donor B cells expressed higher, very uniform amounts of the GL7 marker, intermediate amounts of PNA binding, and lower amounts of sIgD than those observed with the postimmunization protocol. A minor fraction of such cells also appeared to acquire expression of sIgG. We speculate that these differences are due to the high degree of antigen-specific T cell help available to donor B cells as they are incorporated into an already ongoing immune response.

Some B cells participating in the early stages of certain TD immune responses are destined for development into plasmablasts (Liu et al., 1991; Jacob and Kelsoe, 1992; McHeyzer-Williams et al., 1993; Smith et al., 1996; Shih et al., 2002; Pape et al., 2003). Because such cells would not express GL7 and might only express low amounts of B220 but high amounts of

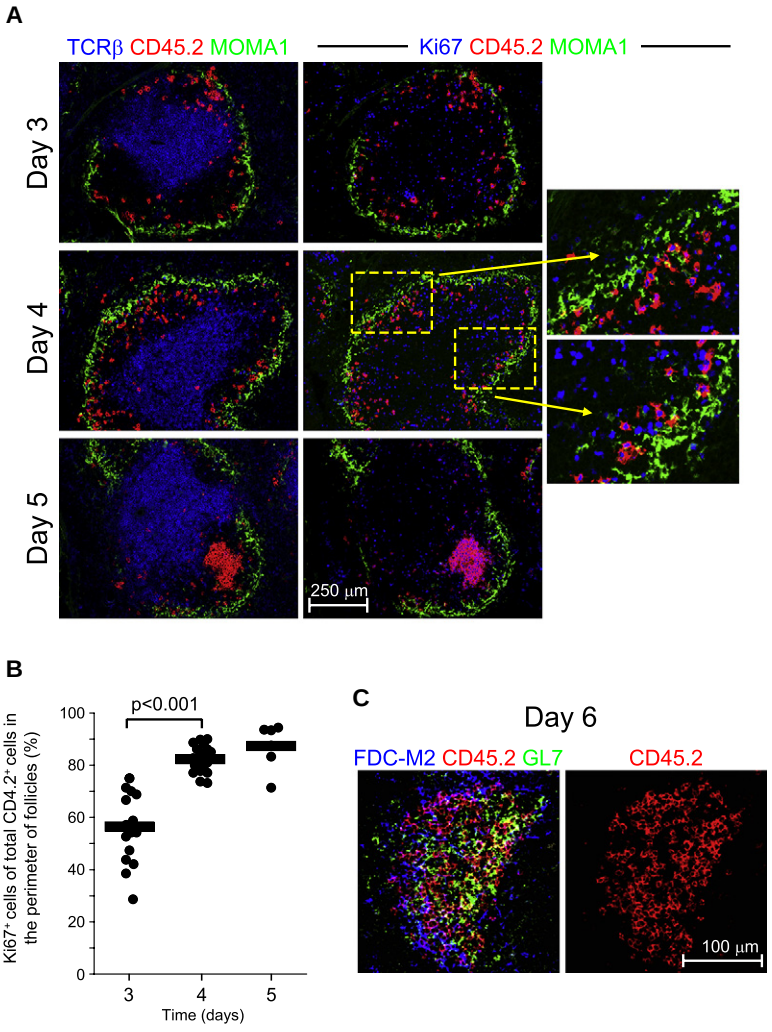

Figure 3. HKI65-Vk10 B Cell Localization to and Proliferation at the Follicular Perimeter Do Not Require Preimmunization

Experiments were performed as described in the legends to Figures 1 and 2 except that recipient mice were not preimmunized. Instead, chimeric mice were immunized 12 hr after cell transfer with Ars-KLH in alum i.p. In all experiments illustrated, 10^5 purified donor HKI65-Vk10 splenic B cells were injected per recipient.

(A) Adjacent spleen sections obtained at the indicated time points after immunization were stained with antibodies specific for the indicated markers. The scales for images in the right and center columns are indicated in the lower panels of each respective column. The images in the right column are magnified regions of areas delineated by the yellow rectangles in day 4, center panel.

(B) Quantitative analysis of CD45.2$^+$ B cell proliferation at the perimeter of follicles on the indicated days. Data obtained represent the percentage of canonical HKI65-Vk10 CD45.2$^+$ B cells that were also Ki67$^+$ counted within 15 cell diameters from the band of MOMA1$^+$ cells that delineate the MZ-follicle border. Each circle represents data from an individual field along this border. Data were obtained from three experiments, in which three to four mice were sacrificed at each time point. A p value was determined with a two-tailed Student's t test.

(C) Spleen sections obtained from chimeric mice 6 days after immunization were stained with antibodies specific for the indicated markers. Data obtained from a representative GC are shown. The scale of the images is indicated in the right panel.

the AFC marker syndecan (CD138), we also monitored the dilution of CFSE in B220$^+$, GL7$^-$, and B220$^-$ and CD138$^+$ donor cells in the chimeric mice. Cell division was only observed in the B220^{high} and CD138$^-$ subpopulations (Figure S1C, top panels; data not shown). Moreover, using both protocols, we did not observe many proliferating donor B cells in the blood during the time of rapid proliferation in the spleen (Figure S2). Taken together with the Ki67 staining studies described above, this argues that most, if not all, of the pre-GC B cell proliferative response take place at the follicular perimeter. Finally, we also investigated the influence of canonical B cell precursor frequency on the results obtained in both protocols. For these studies, HKI65-Vk10 B cells were injected in various doses. As few as 2×10^4 donor B cells could be injected and subsequently detected via histological analyses (see legend to Figure 1 for examples). The results obtained with this small number of cells were analogous to those obtained with larger number of cells.

Canonical B Cells in Ars-KLH Immunized Transgene *Igh*$^{+/-}$ Mice Proliferate at the Follicular Perimeter

HKI65 transgene-targeted hemizygous mice (HKI65 *Igh*$^{+/-}$) have a "semidiverse" BCR repertoire as a result of the expression of endogenous L chain genes. Nonetheless, canonical B cells can be identified in these mice with the anti-clonotypic E4 mAb. To investigate the possibility that localization of proliferating canonical B cells to the follicular perimeter in the studies described above was influenced by the adoptive transfer protocols, we directly immunized HKI65 *Igh*$^{+/-}$ mice with Ars-KLH in alum and performed histological analysis of the canonical B cell response in the spleen at several time points.

As expected, the canonical B cell response in this case was far less uniform and synchronous as compared to that observed with adoptive transfer protocols. Nonetheless, at day 4 after immunization, many E4$^+$ B cells were observed at the follicle-PALS interface, and at day 5 and into day 6, this distribution had changed dramatically in that a major subpopulation of canonical B cells were now observed at the follicular perimeter (Figure S3A). Ki67 staining analysis showed that many of the donor B cells in this locale were proliferating (Figures S3B and S3C). These results strongly indicated that proliferation of pre-GC B cells at the follicular perimeter is not a result of the adoptive transfer protocol used in our studies.

HKI65-Vk10 B Cells at the Perimeter of Follicles Have a Unique Developmental Gene Expression Profile

The above studies suggested that canonical B cells at the perimeter of follicles in immunized mice were intermediates between those initially activated at the T-B interface and GC B cells. We further examined this issue by analyzing the expression of

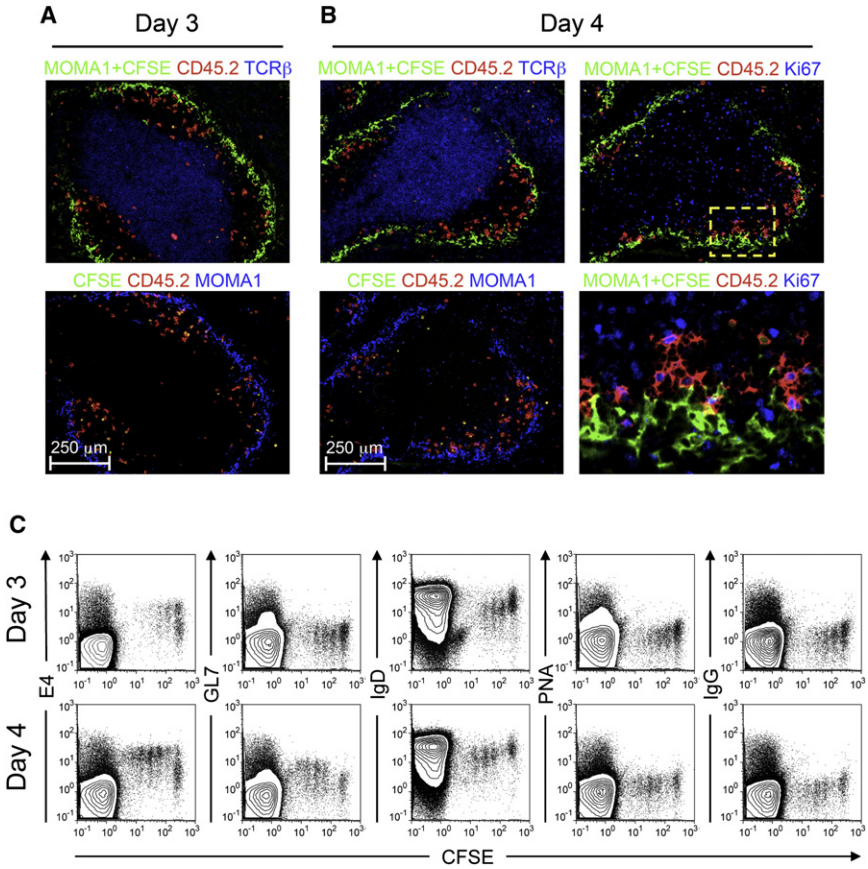

Figure 4. HKI65-Vk10 B Cell Proliferation and Changes in Cell-Surface Phenotype during the Response at the Follicular Perimeter

Chimeric mice were generated and immunized as described in the legend to Figure 3 with CFSE-labeled donor B cells. Spleens isolated at the indicted time points were halved so that one portion could be analyzed by immunofluorescence staining and the other could be analyzed by flow cytometry. In all illustrated experiments, 3×10^6 donor splenic B cells were injected per recipient mouse.

(A) Adjacent spleen sections obtained on day 3 after immunization were stained with Abs specific for the indicated markers and analyzed by immunofluorescence microscopy. The scale for day 3 images is shown in the lower panel.

(B) Images were obtained from adjacent sections of spleens taken on day 4 stained with the indicated Abs. A scale for the left and top right panel is shown in the bottom-left panel. The lower-right panel is a magnified image of the region surrounded by a dashed rectangle in the top-right panel.

(C) Spleen cells were stained with the indicated antibodies or reagents and expression levels of the various markers detected were analyzed by flow cytometry. Dilution of CFSE fluorescence in donor cells was not detected until day 3. The data in this figure are representative of those obtained in three independent experiments in which two to three mice were sacrificed on each day.

various genes required for GC and AFC development and B cell migration via Q-RT-PCR in four subpopulations of B cells purified by FACS: naive B cells from C57BL/6 mice (used as a standard) and three fractions of B cells from chimeric mice at day 3 after donor cell injection of immunized recipient mice: $GL7^-$, $CFSE^+$, (donor B cells that had not divided), $GL7^{int}$, $CFSE^{int}$ (donor cells that had divided four to six times), and $GL7^+$, $CFSE^-$ cells (donor and host GC B cells that had divided extensively, see lower-right inset, Figure 5).

Figures 5A and 5B illustrate that, as predicted from previous studies, $GL7^+$, $CFSE^-$ GC B cells expressed high levels of RNA encoding activation induced cytidine deaminase (AID) (Muramatsu et al., 1999), IgG H chain and Bcl-6 (Onizuka et al., 1995; Cattoretti et al., 1995; Allman et al., 1996; Flenghi et al., 1996), CXCR4 (Allen et al., 2004), and CXCR5 as compared to naive B cells. These cells expressed moderate amounts of BLIMP-1 and nearly undetectable CCR7 RNA. In contrast, $GL7^-$, $CFSE^+$ donor B cells had undetectable RNA encoding AID, low amounts for IgG H chain, modest amounts for Bcl-6, BLIMP-1, and CXCR5, very low amounts for CXCR4, and high levels for CCR7. This expression profile is consistent with B cells that had recently left the interface of B and T cell zones, given that CCR7 is required for B cell migration to this locale (Reif et al., 2002; Cyster, 2005). Cells with a $GL7^{int}$, $CFSE^{int}$ phenotype expressed low amounts of RNA for AID and Bcl-6, and very low to undetectable amounts of RNA for IgG, BLIMP-1, CCR7, CXCR4, and CXCR5. The low expression of RNA for the chemokine receptors indicated that these cells, predominantly located at the perimeter of follicles, were sessile. Histological analyses confirmed that few donor B cells in this locale expressed IgG at the day 3 time point (data not shown). In total, these data support the conclusion that proliferation of cells at the follicular perimeter constitutes a discrete stage in memory B cell development.

B Cell Proliferation at the Perimeter of Follicles Is Not Accompanied by Somatic Hypermutation

To further test this idea, we determined whether donor HKI65-Vk10 B cells that had undergone various numbers of divisions in the chimeric mice had activated SHM to an extent characteristic of GC B cells. B cells present in different CFSE intensity peaks were purified by FACS. The HKI65 V_H locus was PCR amplified from these fractions, and the products were cloned and sequenced. No mutations were found in V_H clones obtained from division peaks zero and five isolated 3 days after donor cell injection (Table S1). In contrast, anticlonotype$^+$ GCs microdissected from sections of spleens isolated at day 5 after donor cell transfer yielded HKI65 PCR V_H clones with an average mutation frequency of 0.4%, and 30% of these mutations were found in SHM "hotspots." These mutations were rather randomly distributed, and those known to increase affinity for Ars were rare (data not shown), as would be expected at this early stage of the GC reaction (Berek et al., 1991; Jacob et al., 1993; McHeyzer-Williams et al., 1993).

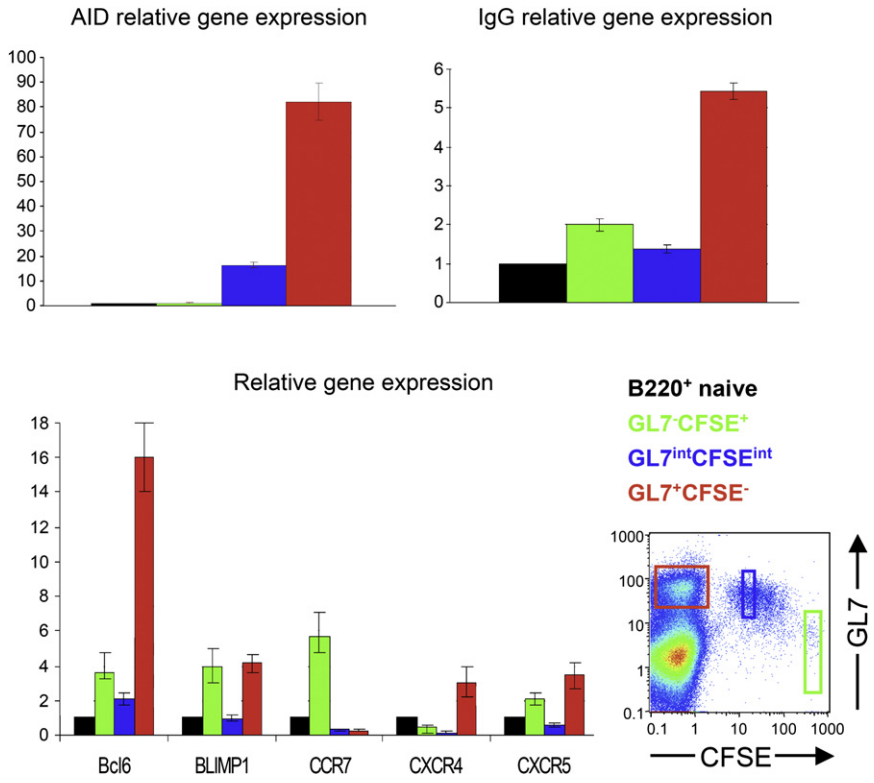

Figure 5. Gene Expression Profile of HKI65-Vk10 Donor B Cells after Different Numbers of Cell Divisions during the Follicular Perimeter Response

Ars-KLH-primed recipient mice were injected with CFSE-labeled splenocytes from HKI65-Vk10 mice (as described in the legend to Figure 1 and Experimental Procedures) and sacrificed on day 3 after transfer. Spleen cells from the chimeric mice were stained with anti-B220 and GL7 Abs, and B cells in different CFSE dilution subpopulations were isolated by FACS (see plot on lower right). QRT-PCR analyses were performed on RNA extracted from $B220^{+}GL7^{int}$, $CFSE^{int}$ cells in division peak 5 (blue), $B220^{+}GL7^{-}$, $CFSE^{+}$ cells in division peak 0 (green), and $B220^{+}GL7^{+}$, $CFSE^{-}$ cells, representing germinal center B cells (red). RNA isolated from B cells of naive C57BL/6 mice (black) served as a reference control whose QRT-PCR value for each assay was arbitrarily set to one. All data are representative of at least two independent experiments. Bar graphs show the mean values (±SD) of triplicate measurements.

These data are consistent with those in Figure 5 showing that low and very high amounts of AID expression were apparent in donor $GL7^{int}$, $CFSE^{int}$, and $GL7^{+}$, $CFSE^{-}$ (GC) B cells, respectively.

Anti-HEL Ig Transgenic B Cells Rapidly Proliferate at the Perimeter of Follicles

We next considered that the rapid proliferative response of pre-GC B cells at the perimeter of follicles might only take place during immune responses to certain antigens. To investigate this possibility, we injected B cells expressing a transgene-encoded BCR specific for hen egg lysozyme (HEL) into immunosufficient, nonirradiated, nontransgenic, syngeneic recipients either primed 3 days earlier with HEL in alum i.p. or 12 hr after cell transfer. Results analogous to those obtained with the HKI65/Ars system were found in this study (Figure 6). Specifically, at days 2 and 3 of the response in the preimmunization protocol (Figure 6A, left and center panels) and day 6 of the postimmunization protocol (Figure 6B, right panels), the majority of donor (IgM^{a+}) B cells and were located at the perimeter of follicles. By day 4 of the preimmunization protocol and day 7 of the postimmunization protocol, many of these donor B cells were becoming $GL7^{+}$ and had entered or formed GCs (Figure 6A, lower-right panel and Figure 6B, day 7). Detailed staining analysis of the GCs obtained with the postimmunization protocol showed that they contained a low frequency of $CD4^{+}$ T cells (Figure 6B, day 7) and extensive FDC networks (Figure 6B, right, bottom panels). Flow cytometric studies using CFSE-labeled anti-HEL B cells revealed that at the time points at which such cells were mainly located at the follicular perimeter, they had divided several times and were $GL7^{+}$ (data not shown). Therefore, anti-HEL Ig transgenic B cells, like HKI65-Vk10 transgenic B cells, undergo rapid proliferation at the follicular perimeter prior to entering the GC reaction.

Location of Accessory Cells during Pre-GC B Cell Proliferation at the Follicular Perimeter

$CD4^{+}$ T cells, FDCs, and certain subclasses of other dendritic cells (DCs) have all been shown to provide growth, survival, and differentiation signals to antigen-activated B cells (Clark and Lane, 1991; Berney et al., 1999; Tew et al., 2001; MacLennan et al., 2003; Haynes, 2008). To determine whether any of these cell types were colocalized at the follicular perimeter during the height of the pre-GC B cell proliferative response there, we performed more extensive histological analyses by using the postimmunization protocol. $CD11c^{+}$ interdigitating DCs were clearly absent from the follicular perimeter (Figure 7A, left panels). Sparsely scattered T cells (Figure 7A, right panels) and FDCs (Figure 7B) were found in this locale, but they did not appear to be directly associated with most donor B cells. Also, staining for the MZ B cell marker CD1d (Figure 7C) suggested that donor B cells had not acquired this phenotype. Analogous results were obtained with the preimmunization protocol (data not shown). These data revealed a scarcity of conventional accessory cells for follicular B cell differentiation and proliferation at the follicular perimeter.

DISCUSSION

The data presented here demonstrate that during the initial phases of GC B cell development, rapid proliferation takes place at the perimeter of follicles. Antigen-activated B cells in this locale

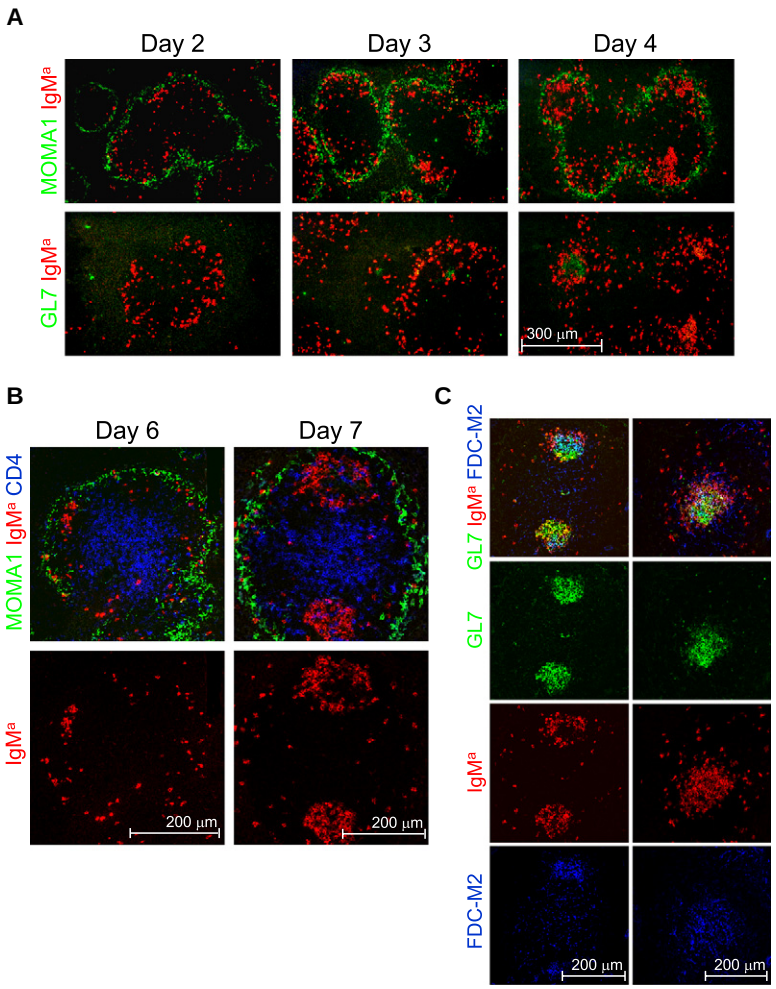

Figure 6. Proliferation and Locale of Anti-HEL Transgenic B Cells during the Immune Response to HEL

(A) Tg(IghelMD4)4Ccg/J splenocytes (10^6 per recipient mouse) were adoptively transferred into nonirradiated C57BL/6 mice primed 3 days earlier with 100 μg of HEL in alum and immediately boosted with 50 μg soluble HEL i.p. On days 2, 3, and 4 after adoptive transfer, donor cell locale was analyzed by immunohistology with Abs specific for the indicated markers. Day 4 images were obtained from adjacent sections. The image scale for all panels is indicated in the lower-right panel. At days 2 and 3 after transfer, donor B cells (red) were seen at the perimeter of follicles and at day 4 they also were detected in GCs. Small $GL7^+$ nascent GCs (green, day 4, lower panel) were detected in some follicles at day 4. The data shown are representative of those obtained from at least two mice at each time point.

(B) Studies analogous to those described for part (A) were performed, but the postimmunization protocol was used. In these experiments, 10^6 donor spleen cells were injected into each recipient mouse. For day 6 and day 7, the lower panels show IgM^a single-color images of the respective upper panels. A scale of the images is indicated in the lower panels.

(C) Panels illustrate donor B cells located within GCs (yellow fluorescence resulting from overlap of IgM^a and GL7 staining) on day 7. Scales for each column of panels are indicated in the bottom panels. The data are representative of images acquired from three separate experiments, in which three to four mice were sacrificed on each day.

display a gene and cell-surface-antigen expression profile consistent with recent departure from the T cell zone (i.e., moderate CCR7 expression prior to proliferation) and ongoing development via the GC pathway (i.e., low to moderate AID, BCL-6, and GL7 expression and sIgD downregulation). Moreover, highly proliferative B cells in this locale express very low levels of RNA encoding CXCR4, CXCR5, and CCR7, indicating that they are not simply "in transit" from the T cell zone to the GC. The subsequent migration of large numbers these B cells to the center of the follicle in which they are resident suggests that GCs are initially formed by B cell coalescence, rather than proliferation in situ.

Our results support previous speculations (Jacob et al., 1993; McHeyzer-Williams et al., 1993) that SHM is not induced during the initial stages of GC B cell proliferation. Past studies on this issue were hampered by lack of knowledge of the time in which GC precursors entered the GC reaction and the degree to which these precursor clones had expanding prior to entry. More importantly, our data indicate that the majority of the "mutation-free" period of pre-GC B cell proliferation takes place at the perimeter of follicles. Very shortly after entering GCs, however, donor B cells in our experiments must have substantially upregulated SHM, given that the majority of the transgenic V_H clones we isolated from microdissected GCs 5 days after cell transfer contained substantial numbers of somatic mutations. These data are incompatible with the idea that high-rate SHM occurs as a function of GC B cell division alone. In contrast, they support the notion that a B cell-extrinsic factor(s) present in the GC proper is required to promote this process.

Our results demonstrating very low or undetectable heavy-chain class switching in B cells rapidly proliferating at the perimeter of follicles contrast with past findings also obtained with a transgenic B cell adoptive transfer approach. In these studies, a major fraction of donor B cells were found to have switched to IgG expression at the T-B interface and in the follicles of the spleen 4 days after immunization and to have undergone more extensive expansion than their IgM-expressing counterparts (Pape et al., 2003). Another previous study suggested that the cytoplasmic tail of membrane IgG confers a proliferative advantage to responding B cells in the AFC pathway (Martin and Goodnow, 2002). Nonetheless, our data demonstrate that expression of an IgG form of the BCR is not a prerequisite for the dramatic burst of proliferation characteristic of the early stages of pre-GC B cell development.

These considerations emphasize the importance of future studies to determine how changes in the many conditions known to affect the quantity and quality of B cell responses impinge upon proliferation and differentiation at the perimeter of follicles. For example, the response of most anti-HEL B cells in this locale was characterized by both rapid proliferation and induction of GL7 and PNA binding, both classical GC B cell markers. In contrast, the proliferative response of HKI65-Vk10 B cells in this locale was slower and accompanied by a more gradual induction of GL7, as well as reduction of IgD expression. It is tempting to speculate that variation in the affinity of the BCR(s)

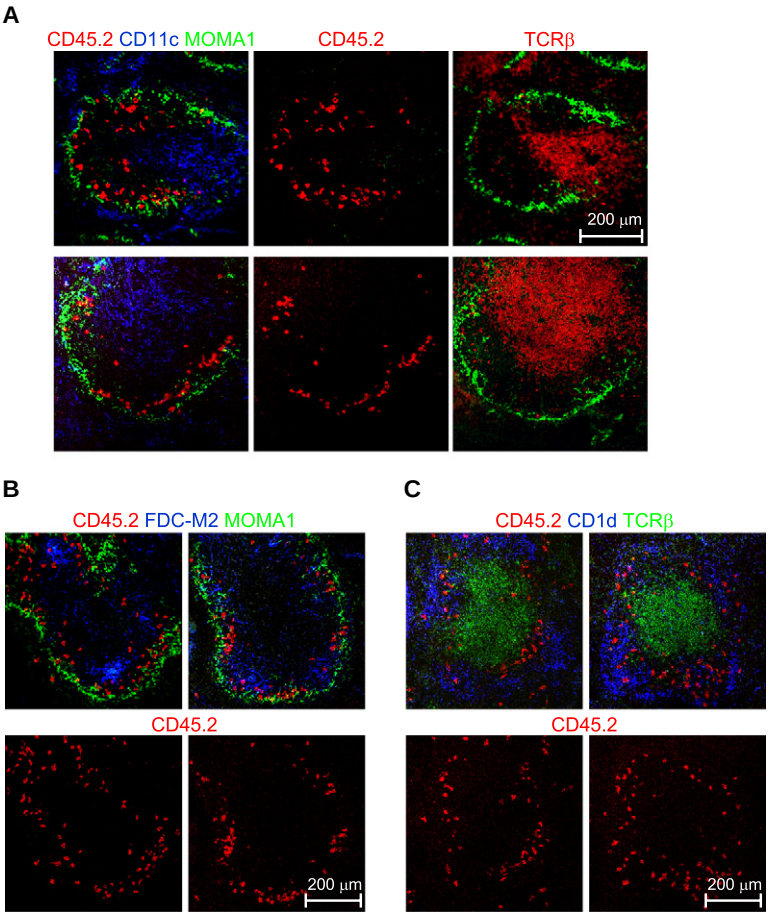

Figure 7. Location of Accessory Cells during the HKI65-Vk10 B Cell Proliferative Response at the Follicular Perimeter

Chimeric mice immunized with Ars-KLH in alum 12 hr after donor cell transfer were sacrificed on day 4 of the response, and spleens were analyzed by immunohistology with Abs specific for the indicated markers. In all experiments, 10^5 purified donor splenic B cells were injected per recipient mouse.

(A) Location of donor B cells, T cells, and CD11c$^+$ DCs. The center column shows single CD45.2 staining from multicolor images acquired for the respective panels in the left column. The scale for all images is indicated in the upper-right panel.

(B) Location of donor B cells and FDCs. Examples of data from two sets of adjacent sections are shown. The image scale is indicated in the lower-right panel.

(C) Location of donor B cells and CD1d$^+$ (mainly MZ) B cells. Data from two sections are illustrated as in (B). Notice the lack of overlap of CD1d (blue) staining and donor (CD45.2, red) staining. The image scale is indicated in the lower-right panel.

expressed by donor B cells for the driving antigen resulted in the differences in cellular behavior described here (e.g., the K_a of the HKI65-Vk10 BCR for Ars is $\sim 2 \times 10^5$ (Rothstein et al., 1983), whereas the K_a of the anti-HEL BCR for HEL is $>10^8$ (Goodnow et al., 1988) and in previous studies.

In total, our results demonstrate that a previously unrecognized microenvironment existing at the perimeter of follicles opposite the T cell zone can promote the initial proliferative stages of the GC B cell response, namely, after primary interaction with T cells at the interface of follicles and the T cell zone, and prior to formation of or entry into the GC. Although our studies were restricted to the spleen, others have observed antigen-specific B cell proliferation in the subcapsular perimeter of lymph node follicles after immunization with a TD antigen (Garside et al., 1998) and have described a unique class of antigen-transporting DCs that localize to this region of LNs shortly after immunization (Berney et al., 1999). Future studies on the factors that regulate the migration of recently activated B cells to and retention and activity of pre-GC B cells in this microenvironment are clearly required.

What might be the function of this intermediate stage of rapid clonal expansion during TD B cell responses in which the structure and function of BCRs remain largely unaltered? In the context of natural infections, Cohn noted many years ago (Cohn, 1972) that unless a large pool of pathogen-specific lymphocytes was generated in a short period of time, the infection would overwhelm the host as a result of the high replicative capacity of most pathogens. Since that time, numerous studies have shown that the GC reaction is an extremely dynamic process in which BCR structures are modified by SHM and CSR, and stringent selective forces are brought to bear on GC B cells on the basis of the affinity and specificity of their BCRs (Kelsoe and Zheng, 1993; MacLennan, 1994; Liu and Arpin, 1997; Camacho et al., 1998). SHM alters the structure of antibody V regions rather randomly (Winter and Gearhart, 1998), and for this reason the majority of somatic mutations are expected to disrupt expression of BCRs, or alter their affinity and specificity such that the GC B cells expressing them are counter selected (Shlomchik et al., 1989; Casson and Manser, 1995; Wiens et al., 1998). Given such considerations, the average rate of SHM per GC B cell division has been argued to approach that which would prevent the clonal expansion of B cells during an immune response (Shlomchik et al., 1989). This suggests that if individual GCs are founded by only small numbers of B cell precursors, subsequent generation of effector populations of sufficient size, expressing BCRs of the affinity and specificity necessary for rapid antigen (pathogen) clearance, might not occur. Therefore, proliferation of pre-GC B cells at the perimeter of the follicle may allow nucleation of the GC reaction by antigen-specific clones large enough to ensure a productive outcome of the subsequent GC response. This scenario would not be inconsistent with the fact that the GC reaction is oligoclonal (Kroese et al., 1987; Jacob et al., 1991; Liu et al., 1991) if the number of B cell precursors to the proliferative phase of the response at the follicular perimeter was limited or if affinity and specificity-based clonal selection took place in this microenvironment.

Limited CSR during this phase of the response may serve a similar function. As discussed above, IgG forms of the BCR appear to have enhanced ability to stimulate proliferation as compared to IgM and IgD forms (Martin and Goodnow, 2002; Pape et al., 2003). Therefore, CSR to IgG isotypes could interfere

with the clonal selection of GC B cells that have increased affinity and that are specific for the driving antigen as a result of SHM. Moreover, delaying the irreversible decision regarding which of the H chain isotypes to express as secreted antibody until after the mutation-selection process is well underway may maximize the probability that highly specific antibodies with effector functions most suited to the nature of the infecting pathogen are generated during TD immune responses. Testing these ideas will require the development of experimental approaches that selectively perturb the migration to and proliferation of B cells at the perimeter of follicles. In this regard, chemokine receptors expressed by responding B cells seem particularly obvious targets. Indeed, Cyster and colleagues (Reif et al., 2002) have observed lodging of CCR7-deficient anti-HEL transgenic B cells at the follicular perimeter after transfer into HEL immunized, CCR7-sufficient recipient mice.

In total, the data we present reveal a previously unappreciated stage of memory B cell development in which rapid clonal expansion takes place in the absence of differentiation at the perimeter of follicles. We suggest that this proliferative phase provides a precursor population for the GC reaction of sufficient size to ensure the subsequent development of the memory B cell compartment. These findings necessitate revision of current models of the TD B cell immune response.

EXPERIMENTAL PROCEDURES

Mice

HKI65 V_H mice have been previously described (Heltemes-Harris et al., 2004). To produce the double-transgenic HKI65-Vk10 mouse line, we bred HKI65 mice to a line of conventional Vk10A light-chain transgenic mice termed Vk1060 (a kind gift of L. Wysocki). Vk1060 transgenic mice bear a functional Vk10A-Jk1 gene encoding the light chain of canonical Ars antibodies. Tg(IghelMD4)4Ccg/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 and B6.SJL-Ptprca Pepcb/BoyJ (B6.CD45.1) mice were originally obtained from the Jackson Laboratory and were bred in house. Mice were housed under pathogen-free conditions and were given autoclaved food and water. The use of mice in these studies was approved by the university's institutional animal care and use committee.

Adoptive Transfers and Immunizations

B cells were enriched from HKI65-Vk10 or Tg(IghelMD4)4Ccg/J spleens via anti-CD4, anti-CD8 and anti-Thy1.2 and C-mediated lysis. Alternatively, non-B cells were depleted by incubation with biotinylated anti-CD3, anti-Thy1.2 and anti-CD11b (Mac-1), or anti-CD43 followed by MACS (Milteny Biotec, Auburn, CA, USA). In some experiments, enriched B cells were labeled with CFSE (Molecular Probes, Eugene, OR, USA) as previously described (Lyons and Parish, 1994). B cells (2×10^4 to 2×10^7) were injected into the tail vein or retro-orbital sinus of syngeneic recipients. Three immunization protocols were used. Recipient mice were either injected with donor cells and immunized 12 hr later with 100 μg of Ars-KLH (or HEL) in alum i.p. (postimmunization protocol) or received 100 μg of Ars-KLH (or HEL) in alum i.p. 1 week or 3 days before cell transfer with an additional injection of 50 μg of Ars-KLH (or HEL) in PBS i.p. at the time of adoptive transfer (preimmunization protocols).

Immunofluorescence

Spleens were snap frozen in OCT compound and cryosections (5–6 μm) made as previously described (Vora et al., 1998). Immunofluorescence staining was also performed as previously described (Heltemes-Harris et al., 2004). The following mAbs and reagents were used: E4-biotin, 5Ci-biotin (both made in house), anti-CD1d-biotin, and anti-TCRβ (H57-597)-biotin (both from BD Biosciences) and then SA-PE (Molecular Probes) or SA-Alexa 633 (Molecular Probes); rat anti-CD169 (MOMA1) (Serotec, Raleigh, NC, USA), rat anti-mouse Ki67 (DakoCytomation, Glostrup, Denmark), FDC-M1, and FDC-M2 (both from ImmunoKontact, Abingdon, UK) and then goat anti-rat IgG-Alexa 633 (Molecular Probes); GL7-FITC, MOMA1-FITC (Serotec); anti-CD11c-FITC; anti-B220 (RA3-6B2)-FITC, anti-TCRβ (H57-597)-PE and FITC, anti-IgM^a-PE, and anti-CD35-PE (all from BD Biosciences); and anti-CD45.2 (104)-PE (eBioscience).

5-Bromo-2′-Deoxyuridine Labeling and Analysis

5-bromo-2′-deoxyuridine (BrdU) labeling was performed with the BrdU Labeling and Detection Kit II from Roche Diagnostics Corporation (Indianapolis, IN, USA) and BrdU (Sigma-Aldrich, St. Louis, MO, USA). In brief, mice were injected i.p. with 1 mg of BrdU and sacrificed 5 or 12 hr later. Spleens were snap frozen in OCT compound and cryosections (5–6 μm) made as previously described (Vora et al., 1998). BrdU detection was done as described in the kit manual. Alternatively, BrdU-labeled cells were revealed with rat BrdU antibodies (Serotec) and then mouse anti-rat IgG-AP (Jackson Immunoresearch Laboratories).

Flow Cytometry

Single-cell suspensions were prepared and stained with combinations of the following Abs and reagents: E4-biotin (made in house), PNA-biotin (Vector Laboratories), GL7, anti-B220 (RA3-6B2)-PE, anti-CD45.2 (104)-PE, anti-CD138 (281.2)-PE (all from BD Biosciences), anti-CD45.2-PE and biotin (eBiosciences), anti-IgD (11-26)-PE (Southern Biotech, Birmingham, AL), and anti-mouse IgG-biotin (Jackson Immunoresearch). Staining with GL7 was followed by staining with biotin-mouse anti-rat IgG (Jackson Immunoresearch Laboratories). Whole-mouse Ig (Jackson Immunoresearch Laboratories) was used for blocking Fc receptors. CyChrome-SA (BD Biosciences), SA-PerCP-Cy5.5, or SA-PE-Cy5.5 (both from eBioscience) were used for detecting biotinylated Abs and other reagents.

Cells were either sorted immediately with an EPICS Elite-ESP or a MoFlo high-performance cell sorter (DakoCytomation) or analyzed on an EPICS XL-MCL (Coulter, Hialeah, FL, USA). If not analyzed immediately, cells were fixed in 2% formaldehyde. Data were analyzed with the FLOWJO software (Treestar, San Carlos, CA, USA).

Molecular Genetics

B cells in peaks of CFSE fluorescence intensity corresponding to different cell divisions were sorted into 1× PBS. Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (QIAGEN). For laser capture microdissection of GCs, frozen spleen sections (7–8 μm) were mounted on PEN-Membrane slides (Leica) and immunohistochemistry was performed as previously described (Vora et al., 1998). In brief, sections were labeled sequentially with anti-CD45.2 (104)-biotin (eBioscience), SA-AP (Vector), and PNA-HRP (Sigma). Slides were developed with the Alkaline Phosphatase Substrate Kit III and NovaRed Substrate kit for peroxidase (both from Vector). Tissue from individual PNA^+, $CD45.2^+$ GCs was microdissected with the LMD6000 laser microdissection system (Leica), and genomic DNA was released by Proteinase K treatment. The HKI65 V_H locus was amplified via nested PCR performed on DNA from sorted cells and microdissected tissue samples with Pfx50 DNA polymerase (Invitrogen). A first round of 35 cycles was performed with the primers 5′-CAACCTATGATCAGTGTCCTC-3′ (hybridizing 5′ of the leader exon) and 5′-GGACTCCACCAACACCATCAC-3′ (hybridizing to the J_H2-J_H3 intron). A small aliquot of the first round was amplified an additional 32 cycles with the primers 5′-CAGGTGTCCACTCTGAGGTTC-3′ (hybridizing to the end of the leader exon and the beginning of the HKI65 V_H locus) and 5′-GTGTCCCTA GTCCTTCATGACC-3′ (hybridizing to the J_H2-J_H3 intron 5′ to the first-round reverse primer). PCR products of the expected size were cloned into plasmid vectors, sequenced at the Kimmel Cancer Center Nucleic Acids Facility and analyzed with CLUSTALW multiple sequence alignment program.

RNA from sorted cells was extracted with the RNeasy mini kit (QIAGEN), according to the manufacturer's instructions and first-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed with the following gene expression assays: Mm 99999055_m1 (*Cxcr4*), Mm00432086 (*Blr1*), Mm01301785_m1 (*Ccr7*), Mm00507774_m1 (*Aicda*), Mm01703609_*g1* (*Ighg*), Mm00477633_m1 (*Bcl6*), Mm00476128 (*Prdm1*), Mm99999915_g1 (*Gapdh*), and universal master mix (Applied Biosystems). Data were collected

with the ABI Prism 7000 sequence detection system (Abbott Diagnostics). Individual gene expression was normalized to *GAPDH*.

Statistical Analysis

Statistical significance was determined with two-tailed, unpaired Student's t test in the Microsoft Excel program.

SUPPLEMENTAL DATA

Supplemental Data include three figures and one table and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00116-2.

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