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Quantitatively Reduced Participation of Anti-Nuclear Antigen B Cells That Down-Regulate B Cell Receptor during Primary Development in the Germinal Center/Memory B Cell Response to Foreign Antigen[1]

Boris Alabyev, Ziaur S. M. Rahman, and Tim Manser[2]

The peripheral B cell compartment contains high levels of "polyreactivity" including autospecificities. We have described a pathway that certain autoreactive B cells may take in gaining stable access to the foreign Ag-responsive peripheral compartment. This pathway was revealed in mice expressing a targeted Ig H chain transgene encoding BCRs with "multireactivity" for the hapten arsonate and DNA-based autoantigens. B cells expressing such BCRs develop to mature follicular phenotype and locale, and are not short-lived. These B cells express very low levels of BCR, indicating that they are not "ignorant" of self Ag, but do not display features of anergy in in vitro assays. Nonetheless, a variety of states of lymphocyte anergy has been described, and some may only be manifested in vivo. As such, we analyzed the ability of these B cells to participate in a T cell-dependent immune response to arsonate in vivo. These B cells mount an early primary response similar to control B cells, including homing to follicles, migration to the T-B interface, and induction of costimulatory molecules, proliferation, differentiation to AFCs, class switching, and entry into GCs and somatic hypermutation. Nonetheless, these B cells display reduced participation in the latter stages of the GC response and in the anamnestic AFC response. In total, these data suggest that while the autoreactivity of this type of B cell does not result in anergy, the ability of such B cells to participate in a cross-reactive immune response to foreign Ag is compromised. ***The Journal of Immunology,*** **2007, 178: 5623–5634.**

B cell tolerance is effected via the action of a variety of "checkpoint" mechanisms operative at various stages of primary and Ag-driven development. Certain types of autoreactive BCRs are removed from the developing repertoire via clonal deletion and receptor editing, mainly in the bone marrow (BM)[3] (1), consistent with Burnet's "forbidden clone" corollary to the clonal selection hypothesis (2). Nonetheless, B cells with particular types of autospecificities can exit the BM and populate the peripheral lymphoid organs. Some of these B cells display phenotypes consistent with "developmental arrest," shortened life spans, blunted activation and differentiative potentials, or fail to gain access to stimulatory lymphoid microenvironments (3–8). Collectively, the state of these cells is often referred to as "anergy" (9–11). Too stringent action of these B cell tolerance pathways via physical or functional deletion of BCRs with any degree of autoreactivity would severely limit the anti-foreign Ag repertoire, as AgRs cannot be monospecific (12–14). In fact, many B cells in peripheral compartments display a "polyreactive" phenotype, including autospecificities (15–18). However, whether and to what extent such B cells are subjected to anergy checkpoints or can participate in foreign Ag-driven immune responses have not been well-studied.

Recently, we described a pathway which certain autoreactive B cells may take in gaining stable access to the periphery. This pathway was revealed during the analysis of mice expressing a targeted Ig H chain transgene encoding Abs with "multireactivity" including binding to the hapten arsonate (Ars) and DNA-based autoantigens such as chromatin (19, 20). Soluble IgG forms of this BCR are analogous to the Abs associated with systemic autoimmune diseases such as lupus in that they have antinuclear staining characteristics (anti-nuclear Ags (ANA)) and cause kidney dysfunction via glomerular deposition in vivo (19). B cells expressing such BCRs develop to mature follicular (FO) phenotype, reside in follicles, and are not short-lived. Nonetheless, these ANA B cells are characterized by substantially reduced levels of BCR (20). These data led us to suggest that during their primary development, such B cells avoid central tolerance and peripheral anergy by reducing their cellular avidity for autoantigen via BCR down-regulation (20, 21). However, this hypothesis was incompatible with other studies showing that reduced BCR levels, particularly of surface IgM (sIgM) are a common characteristic of anergy (1).

For this reason, we previously conducted extensive in vitro analyses of the functional capabilities of these B cells. These analyses did not provide evidence that these B cells were intrinsically anergic (22). For example, these B cells were comparable to nonautoreactive B cells in proliferative responses, induction of activation and costimulatory molecules, and differentiation to Ab-forming cells (AFCs) when treated with a variety of stimuli. Importantly, these ANA B cells were also shown to efficiently process and

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[3] Abbreviations used in this paper: BM, bone marrow; Ars, arsonate; ANA, antinuclear Ag; FO, follicular; AFC, Ab-forming cell; KLH, keyhole limpet hemocyanin; PNA, peanut lectin agglutinin; SA, streptavidin; GC, germinal center; LN, lymph node; FDC, follicular dendritic cell; FR, framework; sIgM, surface IgM; TD, T cell dependent.

present Ag and undergo cognate interaction with naive TCR-transgenic T cells, resulting in robust IL-2 production by the T cells. However, a variety of types of lymphocyte anergy have been reported (23) and it is possible that some forms of anergy are only elaborated in vivo. In this regard, the substantially reduced levels of BCR expressed by these ANA B cells might per se severely limit their in vivo response potential. To address these issues, we have extended our investigations of the functional capabilities of this class of ANA B cells to the Ars-driven, T cell-dependent (TD) immune response in vivo.

Materials and Methods

Mice

HKI65 and HKIR V_H knockin mice have been previously described (19). To produce double-transgenic HKI65/Vk10A and HKIR/Vk10A mouse lines, HKI65 and HKIR mice were bred to Vk10A L chain conventional transgenic mice (a gift of Dr. L. Wysocki, University of Colorado Health Science Center, Denver, CO). Vk10A-transgenic mice bear a functionally rearranged *Vk10* gene encoding the L chain of canonical anti-Ars Abs. C57BL/6 (B6.CD45.2) mice were originally obtained from The Jackson Laboratory and were bred in-house. C57BL/6.SJL (B6.CD45.1) mice were also obtained from The Jackson Laboratory. The M23 bcl-2-transgenic mice (24) were crossed to the A strain for at least 12 generations to generate A.bcl-2 mice. These mice were used to generate HKI65/Bcl-2 and HKIR/Bcl-2 mice by crossing and then backcrossing with HKI65 and HKIR mice, respectively. Mice were housed under pathogen-free conditions and given autoclaved food and water. The use of mice in these studies was approved by the Animal Care and Use Committee.

Adoptive transfers and immunizations

B cells were enriched from HKI65/Vk10 and HKIR/Vk10 spleens by depletion of non-B cells using biotinylated anti-CD3ε, anti-Thy1.2, anti-F4/80, anti-Gr1, and anti-CD11b (Mac-1) followed by MACS (Miltenyi Biotec). In some experiments, enriched B cells were labeled with CFSE (Molecular Probes) as previously described (25). B cells (2×10^4–10^7) were injected into the tail vein of syngeneic B6.CD45.2 or B6.CD45.1 recipients. Two immunization protocols were used. Recipient mice were either adoptively transferred and immunized 12 h later with 100 μg of Ars-keyhole limpet hemocyanin (KLH) in alum i.p. (referred to as protocol B) or received 100 μg of Ars-KLH in alum i.p. 1 wk before transfer with an additional injection of 50 μg of Ars-KLH in PBS i.p. at the time of adoptive transfer (referred to as protocol A).

Immunochemistry and immunofluorescence

Spleens were snap-frozen in Tissue-Tek OCT compound (Sakura Finetek) and cryosections (5–6 μm) were made as previously described (26). Immunohistochemistry and immunofluorescence staining were also performed as previously described (26). The following mAbs and reagents were used: E4-biotin (made in-house), peanut lectin agglutinin (PNA)-HRP (Sigma-Aldrich), anti-B220-PE, anti-B220-FITC, anti-TCRβ-FITC, anti-CD45.2-biotin, anti-CD45.2-PE, anti-GL7-FITC (all BD Biosciences), purified anti-follicular dendritic cell (FDC)-M2 (ImmunoKontact), MOMA1 purified or MOMA1-FITC (Serotec). Purified MOMA1 and FDC-M2 Abs were followed by mouse anti-rat IgG-Alexa 633 (Molecular Probes). All biotinylated Abs were followed by streptavidin (SA)-AP (Vector Laboratories) or SA-PE (BD Biosciences). AP and HRP were detected using the Alkaline Phosphatase Substrate kit III and the Vector NovaRed Substrate kit, respectively (both Vector Laboratories).

Flow cytometry

Single-cell suspensions were prepared and stained with E4-biotin (made in-house), anti-B220-PE, anti-B220-PE-Texas Red, anti-CD45.2-PE, anti-CD45.2-bio (all BD Biosciences), anti-IgD-PE (Southern Biotechnology Associates), PNA-FITC, anti-IgM-FITC (both from Vector Laboratories). CyChrome-SA (BD Biosciences) was used to detect biotinylated Abs. Cells were assayed on a Coulter EPICS XL-MCL analyzer. Data were analyzed using FlowJo software (Tree Star).

ELISPOT assays

Multiscreen 96-well plates (Millipore) were coated with 50 μl of 10 μg/ml of either anti-mouse IgM or anti-mouse IgG (Caltag Laboratories) overnight at 4°C. Splenocytes or BM cells were added in serial dilution to the plates and incubated in RPMI 1640 medium containing 10% FCS for 6 h at 37°C. Biotin-conjugated E4 Abs were added, followed by 50 μl of SA conjugated to AP (Vector Laboratories) at a 1/500 dilution. The plates were developed using the Alkaline Phosphatase Substrate III kit (Vector Laboratories). Spots were counted using a computerized imaging video system (Cellular Technology).

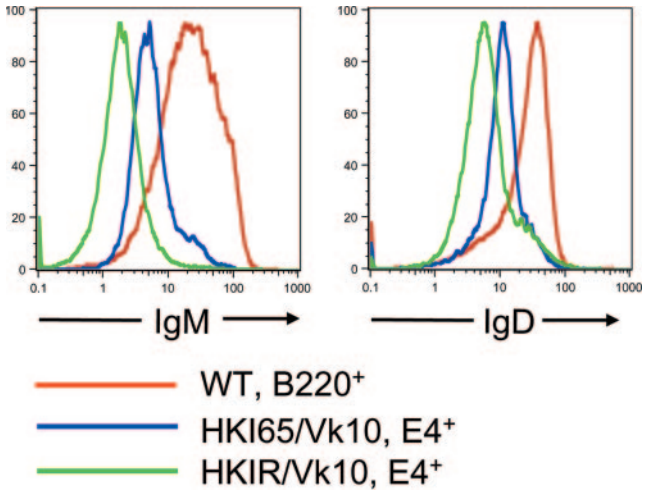

FIGURE 1. Autoreactive HKIR/Vk10 $E4^+$ B cells have reduced surface levels of IgM and IgD. Splenic B cells from naive HKI65/Vk10 and HKIR/Vk10 mice were stained with E4, anti-IgM, and anti-IgD Abs and analyzed by flow cytometry. Surface IgM and IgD levels were measured on E4 gated cells. Wild type (WT) refers to C57BL/6 mice. WT cells were stained with anti-B220, anti-IgM, and anti-IgD Abs and surface BCR levels were measured on B220 gated cells. The histograms shown are representative of results from at least three independent experiments.

ELISA

Ag (anti-Ars), DNA, and clonotype-specific (E4) serum Igs were measured by solid-phase ELISA on 96-well plates (Immulon-4; Thermo Electron) as previously described (27).

Apoptosis assay

Apoptosis was detected in $PNA^+CD45.2^+$ gated cells using the Annexin V-PE Apoptosis Detection kit I (BD Biosciences). Data were analyzed using FlowJo software.

Molecular genetics

Spleen sections were stained with PNA and E4 and tissue from individual PNA^+E4^+ germinal centers (GCs) were microdissected into 1× PCR buffer. Genomic DNA was released by proteinase K treatment with subsequent heat inactivation of enzyme and the V_H knockin locus was amplified via nested PCR. For the first round of 40 cycles, the primers 5′-CAAC CTATGATCAGTGTCCTC-3′ (hybridizing 5′ of the leader exon) and 5′-GAAATGCAAATTACCCAGGTG-3′ (hybridizing to the J_H2-J_H3 intron) were used. A small aliquot of the first round reaction was amplified for 40 additional cycles using the primers 5′-CAGGTGTCCACTCTGAG GTTC-3′ (hybridizing to the end of leader exon and the beginning of the V_H gene), and 5′-GTGTCCCTAGTCCTTCATGACC-3′ (hybridizing to the J_H2-J_H3 intron 5′ to the first round reverse primer). PCR products of the expected size were cloned using the pGEM T-easy vector system (Promega), sequenced at the Kimmel Cancer Center Nucleic Acids Facility, and analyzed using the CLUSTALW multiple sequence alignment program (http://www.ebi.ac.uk/clustalw/).

Results

The ANA B cells discussed above develop in a previously described line of V_H knockin mice, termed HKIR, in which the transgenic V_H gene contains a single mutation to arginine (R) at position 55 in the CDR2 subregion (19, 20). Another line of V_H knockin mice, termed HKI65, contains an Ig knockin locus identical with that in the HKIR line, but the transgenic V_H gene lacks the position 55 R mutation (20). Both versions of this V_H gene, in

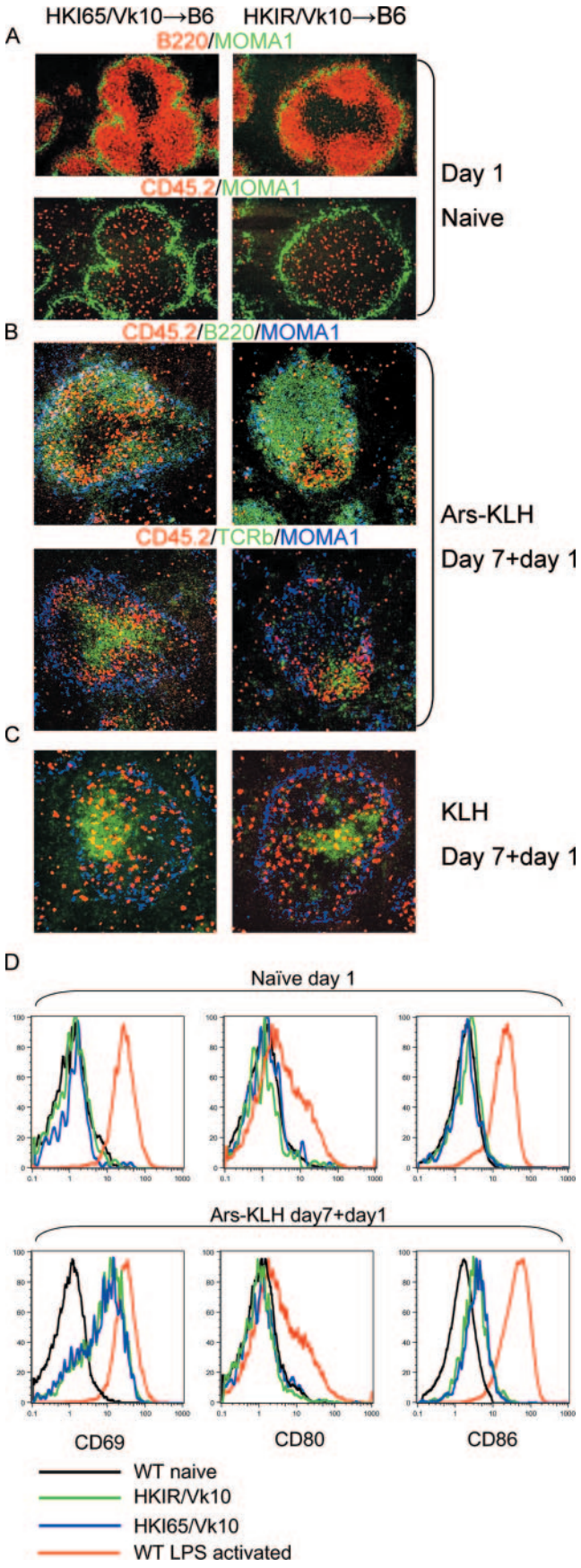

FIGURE 2. Localization and activation of HKI65/Vk10 and HKIR/Vk10 B cells in naive and immunized chimeric mice. *A*, Serial spleen sections from naive B6.CD45.1 recipients injected with either HKI65/Vk10 or HKIR/Vk10 splenocytes were stained with either anti-B220 (red) and MOMA1 (green) (*upper panel*) or anti-CD45.2 (red) and MOMA1 (green) (*lower panel*). Flow cytometry analysis revealed 90% of CD45.2$^+$ cells in the spleens of these mice were B220$^+$. *B*, Localization of donor HKI65/Vk10 and HKIR/Vk10 B cells at the interface of T and B cell zones in Ars-KLH immunized mice. Serial spleen sections from B6.CD45.1 recipients injected with either HKI65/Vk10 or HKIR/Vk10 B cells according to protocol A and sacrificed the next day were analyzed. *Upper panel*, The B cell zone was revealed by staining with anti-B220 (green). MOMA1 staining was used to delineate the white pulp border and appears in blue. Donor B cells (red) were stained with anti-CD45.2. *Lower panel*, The T cell zone was revealed by staining with anti-TCRβ (green), MOMA1 staining appears in blue, and anti-CD45.2 staining appears in red. *C*, Localization of donor B cells when KLH was used as an Ag (protocol A). Anti-TCRβ (green), MOMA1 (blue), and anti-CD45.2 (red) staining was performed. *D*, Levels of activation markers on HKI65/Vk10 and HKIR/Vk10 E4$^+$ B cells. HKI65/Vk10 or HKIR/Vk10 splenic B cells were adoptively transferred into naive B6 recipients (*upper panel*) or B6 recipients primed with Ars-KLH in alum 1 wk earlier (protocol A, *lower panel*). Eighteen hours later, splenocytes from recipient mice were stained for the indicated markers and analyzed by flow cytometry. B cells from WT (C57BL/6) controls were B220 gated; whereas HKI65/Vk10- and HKIR/Vk10-derived cells were gated on B220 and E4. LPS-activated splenocytes from WT (B6) mice were used as a positive control. The histograms shown are representative of results from at least three independent experiments.

combination with a single, endogenous κ L chain gene, encode Abs that we term canonical. Both types of canonical Abs bind the hapten Ars with indistinguishable affinity and can be detected using the monoclonal anti-clonotypic Ab E4 (19, 20). However, these canonical Abs differ in their affinity for DNA-based autoantigens. The HKIR form stains nuclei and mitotic bodies in ANA assays, but the HKI65 form does not, and the HKIR form displays ~10-fold higher apparent affinity for ssDNA and dsDNA than the HKI65 form in ELISA (19). Canonical HKI65 BCRs are identical with those previously shown to be expressed by primary B cells which give rise to progeny that dominate the memory response of A/J mice to Ars (28).

Our previous studies have shown that canonical B cells are present in similar numbers in the splenic and lymph node (LN) follicles of HK65 and HKIR mice and–with the exception of the somewhat and dramatically reduced levels of BCR they express, respectively–have the phenotypic characteristics of mature FO B cells (20, 22). To determine whether canonical HKIR and HKI65 B cells differed in their ability to respond to immunization with a TD Ag, mature B cell adoptive transfer protocols were used (29, 30). The use of this approach reduced the possibilities that immature B cells, that might not yet have encountered autoantigen, could be recruited into the response (>95% of canonical peripheral B cells in both strains have a mature FO phenotype), and that an unusually high precursor frequency of transgenic V region expressing B cells would affect the B cell response.

To increase the precursor frequency of canonical clonotypes in the donor B cell compartment for many of these studies, the HKIR and HKI65 lines (backcrossed for >15 generations to the C57BL/6 (B6) background) were crossed to a previously described line of conventional Ig κ-transgenic mice (also on a B6 background, Ref. 31), expressing the Vk10A-Jk1 L chain that combines with canonical Ig H chains to form canonical Abs and BCRs. Extensive analysis of the primary development of E4$^+$ canonical clonotypes in the resulting double transgenic mice (termed HKIR/Vk10 and HKI65/Vk10) revealed no differences from those previously published (20, 22) for canonical HKIR and HKI65 clonotypes expressing endogenous Vk10A-Jk1 L chains (data not shown). Importantly, most, if not all of these clonotypes were located in follicles and were of FO phenotype in spleen and LN (in spleen >95% of

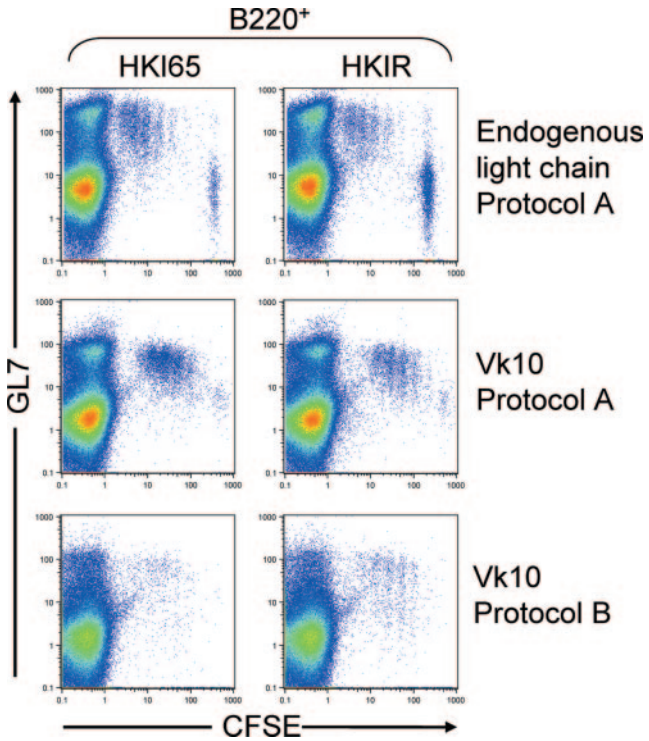

FIGURE 3. Proliferation of HKI65 and HKIR B cells during the early stages of the TD immune response to Ars. HKI65$^{+/-}$ and HKIR$^{+/-}$ or HKI65/Vk10 and HKIR/Vk10 B cells were purified via MACS, labeled with CFSE, and injected separately i.v. into nonirradiated syngeneic recipients according to protocol A (*two upper panels*) or protocol B (*lower panel*) as described in *Materials and Methods*. On days 3 (protocol A) and 5 (protocol B) after transfer, splenocytes from chimeric mice were collected, stained with anti-B220 and GL7, and analyzed by flow cytometry. Cells were B220 gated and the extent of CFSE dye dilution corresponding to various numbers of cell divisions was analyzed.

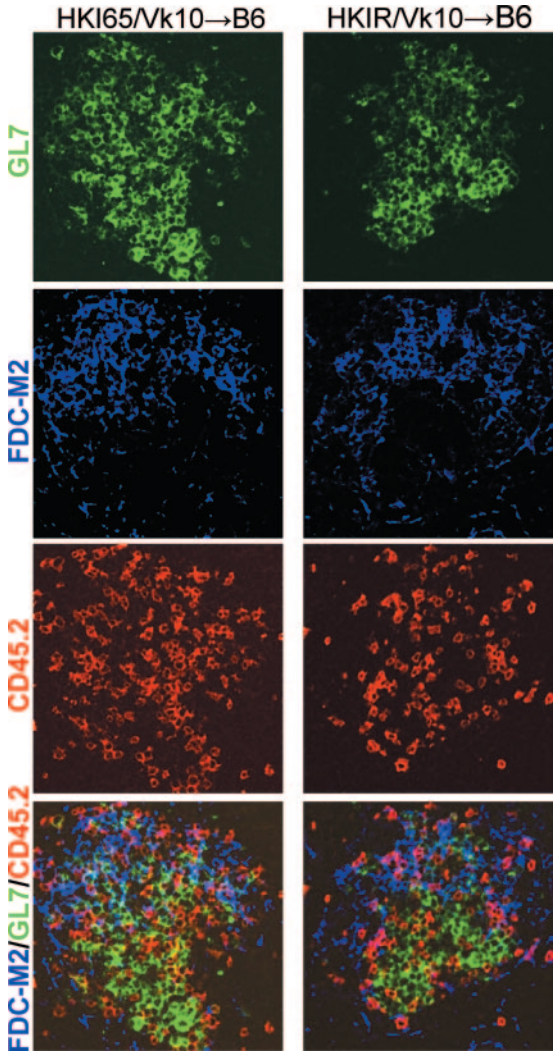

FIGURE 4. Most proliferating HKI65/Vk10 and HKIR/Vk10 B cells reside in GCs. HKI65/Vk10 and HKIR/Vk10 B cells were adoptively transferred into syngeneic B6.CD45.1 recipients according to protocol A and sacrificed on day 5 after transfer. Spleen sections were stained for GL7 (green) and FDC-M2 (blue) to define germinal centers and for CD45.2 (red) to define donor B cells. Images of single GCs of each type are illustrated. Merged images are shown on the bottom panel. Original magnification was ×360.

all B cells express canonical, E4$^+$ BCRs), and the low and intermediate levels of sIgM and sIgD characteristic of canonical clonotypes in HKIR and HKI65 mice, respectively, were recapitulated in HKIR/Vk10 and HKI65/Vk10 mice (Fig. 1). In addition, neither HKIR/Vk10 nor HKI65/Vk10 clonotypes displayed any evidence of an anergic phenotype in in vitro studies analogous to those previously conducted on canonical clonotypes in HKIR and HKI65 mice (53).

Canonical HKIR B cells home to follicles, up-regulate costimulatory molecules, and proliferate at a rate similar to canonical HKI65 B cells in response to Ars

We followed previously established adoptive transfer protocols designed to study TD immune responses generated by Ig-transgenic B cells (29, 30). To facilitate the ability to distinguish donor from host cells, the CD45.1/.2 allotype system was used. To evaluate homing of HKI/Vk10 B cells (CD45.2$^+$), unirradiated, naive C57BL/6.CD45.1 (B6) mice were injected i.v. with equal numbers of splenic B cells from HKI65/Vk10 and HKIR/Vk10 mice and histological analysis of the spleens of the chimeric mice was performed 1 day later. Fig. 2*A* shows that both types of B cell homed equally well to the spleen and were predominately located in white pulp areas. Next, splenic B cells from HKI/Vk10 mice were injected i.v. into B6.CD45.1 mice that had been immunized with Ars-KLH on alum i.p. 1 wk earlier, followed by a single injection of soluble Ars-KLH i.p. immediately after cell transfer (termed protocol A). At various times thereafter, the chimeric mice were sacrificed and spleens harvested for flow cytometric and histological analysis. Recent reports have indicated that the results obtained from such an adoptive transfer approach may vary with the dose of donor lymphocytes (32). As such, most of our experiments were performed using doses of canonical donor B cells per recipient mouse ranging from 5×10^5 to 5×10^4 and qualitatively similar results were obtained.

Fig. 2*B* shows that 1 day after transfer, many donor B cells were observed at the interface of B cell and T cell zones, the site of initial cognate T-B interaction in the spleen (33–35). Analogous results were obtained in HKIR→B6 and HKI65→B6 chimeras (data not shown). In contrast, when KLH was used as the Ag in the same protocol, donor HKI65/Vk10 and HKIR/Vk10 B cells were observed to be rather randomly distributed, mainly in white pulp regions (Fig. 2*C*). These data demonstrate that interaction of HKI65 and HKIR donor B cells with cognate Ag is required to induce migration to the T-B interface.

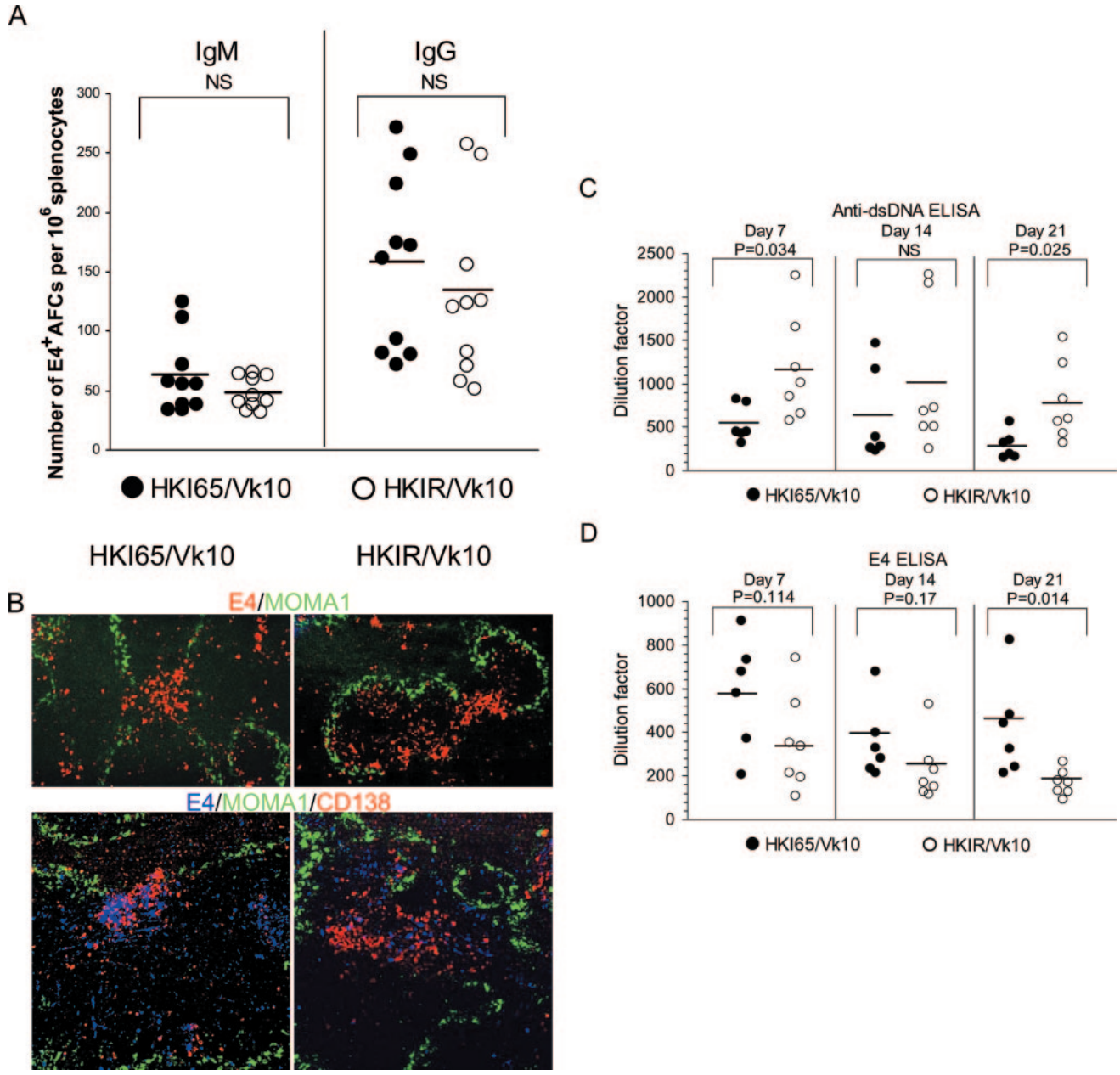

FIGURE 5. Frequency and location of $E4^+$ AFCs and Ab titers in immunized HKI65/Vk10 and HKIR/Vk10 chimeric mice. *A*, Chimeric mice were created according to protocol B as described in *Materials and Methods.* On day 6 after immunization with Ars-KLH, recipient spleens were analyzed by ELISPOT and the number of $E4^+$ IgM- and IgG-producing AFCs per million splenocytes was counted. Horizontal bars represent mean values for each data set. ● and ○, Number of $E4^+$ AFCs produced in duplicate assays by individual HKI65/Vk10 and HKIR/Vk10 chimeric mice, respectively. Statistical significance was assessed using the Student *t* test. Five mice of each genotype were analyzed. *B*, Sections of the same spleens used for the ELISPOT analysis were stained with E4 (red) and MOMA1 (green) (*upper panel*), or E4 (blue), MOMA1 (green), and CD138 (syndecan) (red) (*lower panel*). Original magnification was ×250. *C* and *D*, HKI65/Vk10 and HKIR/Vk10 chimeric mice were created according to protocol B and bled on days 7, 14, and 21. E4 specific ELISA (*C*) or anti-dsDNA ELISA (*D*) of serum samples were performed on serum taken at the indicated days. Values represent the serum dilution factor at which the OD_{405} reached the value of 0.9 (*C*) or 0.5 (*D*). Each circle represents data from an individual mouse and at least six mice were analyzed for each time point. Horizontal bars represent mean values for each data set. Statistical significance was assessed by the Student *t* test.

The homogeneity of the donor B cell response in HKIR/Vk10→B6 and HKI65/Vk10→B6 chimeras allowed flow cytometric assessment of the induction of activation of costimulatory molecules associated with T cell-dependent B cell activation on canonical B cells. Fig. 2*D* shows that levels of CD69 and CD86 had been significantly and homogenously up-regulated on both HKI65/Vk10 and HKIR/Vk10 donor B cells 1 day after transfer into Ars-immunized, but not naive, recipients. CD80, a costimulatory molecule known to be more slowly induced (36), did not differ in levels of expression from naive controls.

To evaluate levels of proliferation, donor B cells were labeled with CFSE and flow cytometric analysis of splenic B cells performed at various times after cell transfer. At day 1, little or no cell division was detected in Ars-KLH-immunized chimeras (data not shown), but on day 3 a major subpopulation of donor B cells in HKIR→B6 and HKI65→B6 mice had undergone five to six divisions (approximately one division every 8–9 h on average) and acquired the activation marker GL7 (Fig. 3, *upper panels*). Examination of HKIR/Vk10→B6 and HKI65/Vk10→B6 chimeras allowed specific determination of whether canonical clonotypes displayed this

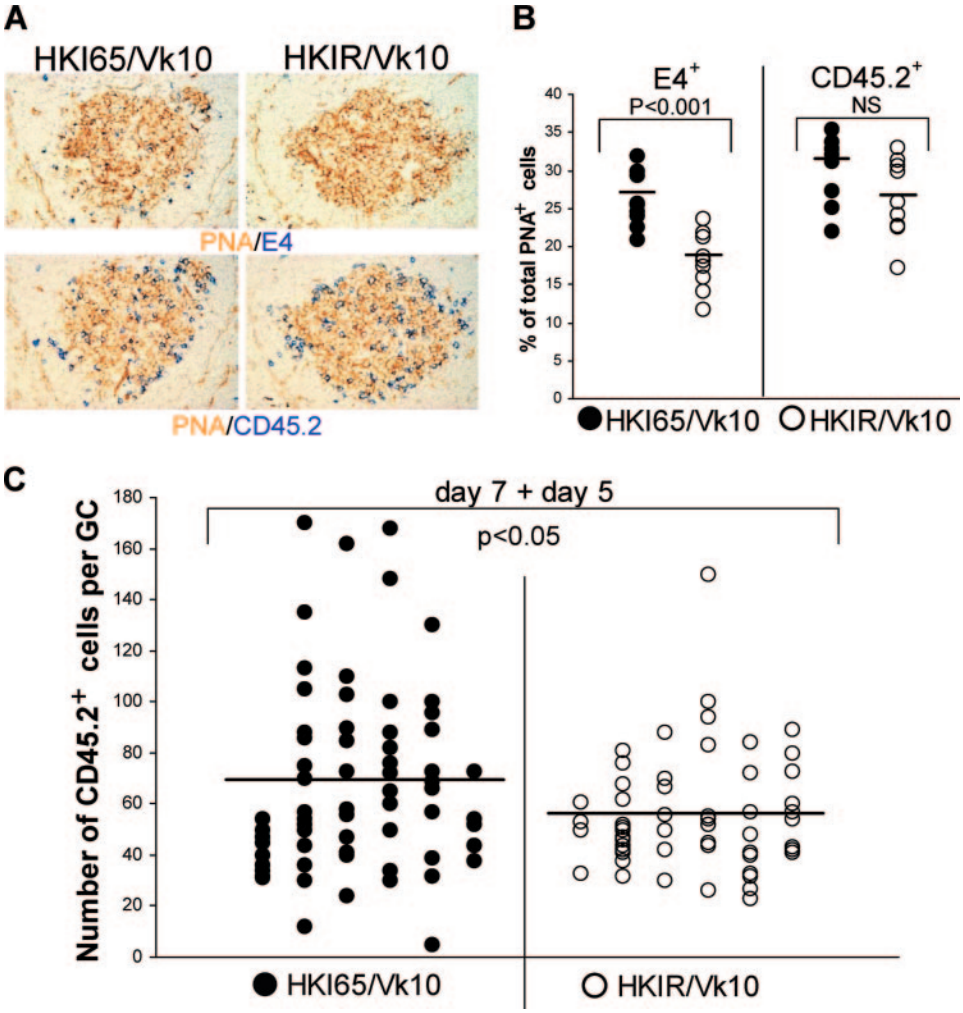

FIGURE 6. HKIR/Vk10 B cells populate GCs. HKI65/Vk10 and HKIR/Vk10 chimeric mice were created according to protocol A as described in *Materials and Methods.* Mice were sacrificed on day 5 after adoptive transfer. *A*, Adjacent cryosections of mouse spleens were stained with PNA (brown) and either E4 (*upper panel*, blue) or anti-CD45.2 (*lower panel*, blue). *B*, The percentage of $E4^+$, PNA^+ cells from the total number of $B220^+$, PNA^+ gated B cells in HKI65/Vk10 (●) and HKIR/Vk10 (○) chimeric mice at day 5 after cell transfer (protocol A) was assessed by flow cytometry. Each circle represents data obtained from an individual mouse. *C*, Number of donor derived $CD45.2^+$ cells per $CD45.2^+$ GC as assessed by histology. At least 10 randomly selected GCs of medium size (20–30 cell diameters) from each mouse were analyzed. ● and ○, The number of HKI65/Vk10 $CD45.2^+$ and HKIR/Vk10 $CD45.2^+$ cells per GC, respectively. Each circle represents an individual GC and each group of circles represents data from an individual mouse. Horizontal bars represent mean values for each data set. Statistical significance was determined using the Student *t* test.

same behavior. At day 3, most donor cells in these chimeric mice had become $GL7^+$ and had undergone four to five divisions (average division rate ~9–10 h, Fig. 3, *middle panels*). Staining with the E4 Ab in these studies revealed that essentially all canonical donor B cells had been recruited into the proliferative response (data not shown). Control experiments in which KLH was used as Ag in this protocol revealed no evidence of cell division in donor B cell populations at any of these time points (data not shown).

To determine whether this robust and equivalent participation of canonical HKIR and HKI65 clonotypes in the proliferative response was also apparent in a situation where recipient mice had not been preimmunized with Ars, another protocol (B), in which CFSE-labeled donor B cells were transferred to naive recipients that were immunized 12 h later with Ars-KLH in alum i.p. was used. This resulted in a slower and less synchronous donor cell response as compared with protocol A (data not shown). Nonetheless, Fig. 3, *lower panels*, illustrates that most HKIR/Vk10 and HKI65/Vk10 clonotypes had divided five to six times 5 days after immunization of chimeric mice (average division rate ~20–24 h). Acquisition of the GL7 marker by proliferating B cells was somewhat less extensive in protocol B than in protocol A, but the majority of proliferative donor cells was nonetheless $GL7^+$ in both protocols.

Histological analysis of spleen sections from mice at the same time points in which flow cytometric analysis indicated extensive proliferation showed that many $E4^+$ (and IgM^{a+} or $CD45.2^+$) donor B cells lacked detectable CFSE fluorescence, presumably due to the relative insensitivity of fluorescence microscopy as compared with flow cytometry (data not shown). As such, we evaluated the locale of proliferating HKI65/Vk10 and HKIR/Vk10 B cells using CD45.2, GL7, and FDC-M2 (to detect follicular dendritic cells) three-color staining. The majority of $CD45.2^+$ B cells in both types of chimeras were located in GCs at the day 5 time point (protocol A, Fig. 4). In addition, these donor B cells were approximately equally distributed in FDC-rich and FDC-poor areas of the GC.

Comparable primary IgM and IgG AFC responses from canonical HKIR and HKI65 clonotypes

Our previous studies have shown that canonical HKIR B clonotypes are capable of efficiently differentiating to IgM- and IgG-producing AFCs when stimulated with LPS or anti-IgM and IL-4 in vitro (22). To evaluate the ability of these clonotypes to participate in the Ars-driven primary AFC response in vivo, transfer protocol B was used, as preliminary studies on HKIR/Vk10 B cells indicated that this protocol produced a far more vigorous AFC response than protocol A. Fig. 5*A* shows that 6 days after cell transfer and immunization, comparable numbers of $E4^+$ splenic AFCs of both IgM and IgG isotypes were detected in HKIR/Vk10→B6 and HKI65/Vk10→B6 chimeric mice. Fig. 5*B* illustrates that at this same time point intensely stained $E4^+$ cells were located in the white pulp bridging channels, in areas containing numerous CD138 (syndecan)$^+$ cells, as expected of a normal primary AFC response.

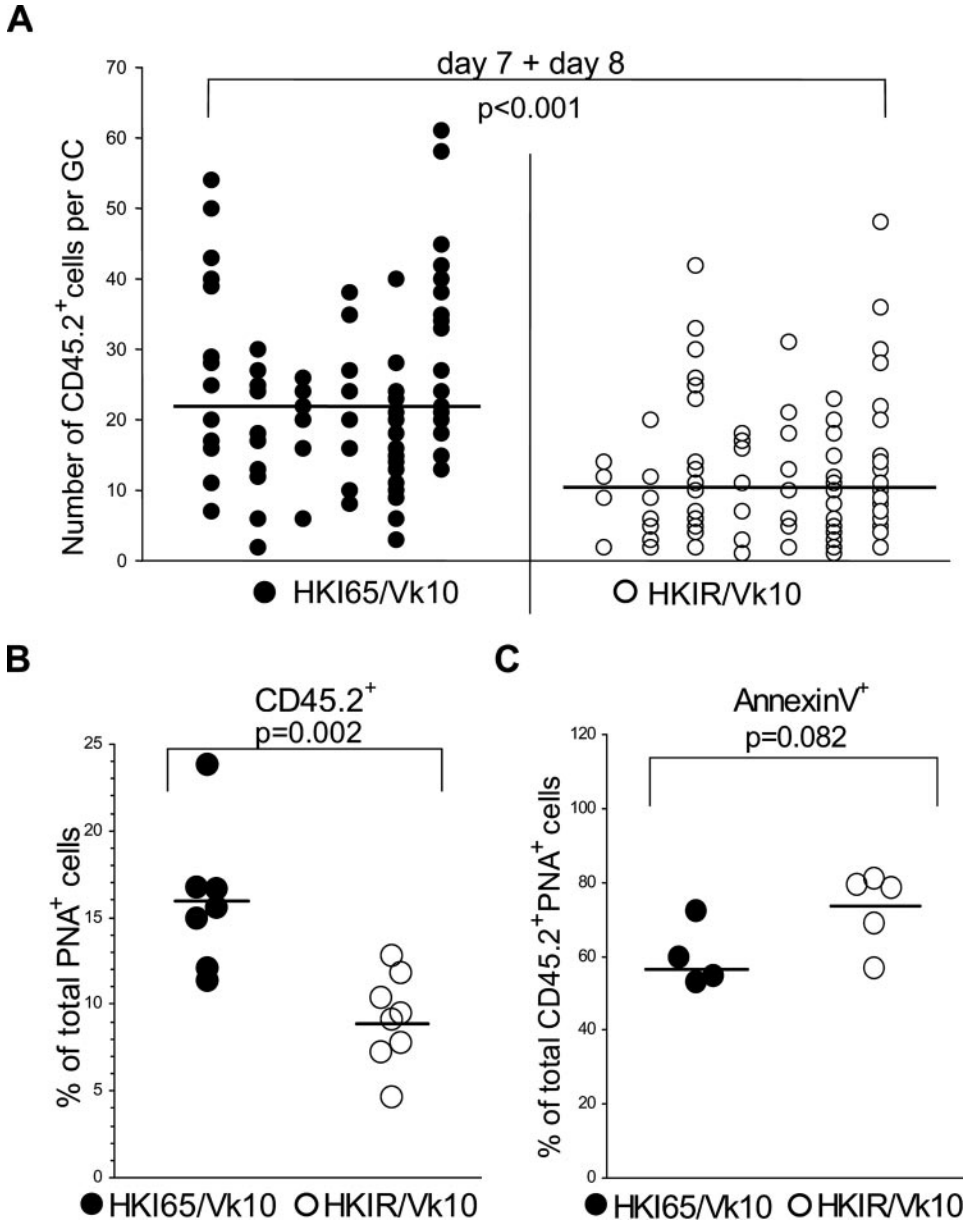

FIGURE 7. HKIR/Vk10 GC B cells are less frequent and reveal higher levels of apoptosis than HKI65/Vk10 GCs B cells at later stages of the GC response. *A*, The experiment was conducted and the data are presented in the same way as described in the legend to Fig. 6*C*, except that spleens were harvested on day 8 after adoptive transfer. At least 10 randomly selected GCs of small size (10–20 cells in diameter, medium size GCs were rare at this time point) from each mouse were analyzed. *B*, At the same time point, splenocytes were stained for B220, PNA, and the CD45.2 allotypic marker and analyzed by flow cytometry. ● and ○, The percentage of HKI65/Vk10 CD45.2$^+$ and HKIR/Vk10 CD45.2$^+$ cells of total B220$^+$ PNA$^+$ cells analyzed in each mouse, respectively. *C*, The same cells analyzed in *B* were stained with CD45.2, PNA, and annexin V and analyzed by flow cytometry. ● and ○, The percentage of HKI65/Vk10, annexin V$^+$, and HKIR/Vk10, annexin V$^+$ cells of total PNA$^+$CD45.2$^+$ cells analyzed, respectively. Each circle represents data from an individual mouse. Horizontal bars represent mean values for each experimental set. Background levels of apoptotic B220$^+$ cells were evaluated in naive B6 mice and yielded an average value of 16%. Statistical significance was assessed by the Student *t* test.

These data indicated that HKIR/Vk10 B cells were capable of producing a primary serum Ab response. Indeed, Fig. 5*C* shows that robust levels of E4$^+$ serum Abs were detected at day 7 after cell transfer in HKIR/Vk10→B6 chimeras. However, these levels were somewhat lower than those produced in HKI65/Vk10 chimeras. Moreover, the levels of serum E4$^+$ Abs in HKIR/Vk10→B6 chimeras decreased rapidly after day 7. Whether this is due to more rapid clearance of these autoreactive Abs will require further investigation. This decline does not appear to result from a selective defect in the ability of canonical HKIR B cells to generate long-lived AFCs, as analysis of splenic and BM E4$^+$ AFC levels in both HKIR→B6 and HKI65→B6 chimeras at late times after transfer revealed undetectable levels of such AFCs (data not shown).

Fig. 5*D* shows that, as expected, levels of serum anti-DNA Abs were elevated in HKIR/Vk10→B6 as compared with HKI65/Vk10→B6 chimeras. However, by day 21 these levels had declined. At this, and at later time points after immunization, HKIRVk10→B6 chimeric mice showed no overt signs of autoimmune disease, consistent with the notion that foreign Ag drive is required to sustain the AFC response of HKIR/Vk10 B cells.

Progressively diminished germinal center responses from canonical HKIR clonotypes

Critical steps in the TD B cell immune response leading to memory take place in the GC. As such, we next evaluated the recruitment of canonical HKIR and HKI65 clonotypes into the GC reaction. At day 5 post-donor cell transfer (protocol A) numerous E4$^+$ B cells could be observed in GCs of chimeric mice generated with HKI65 and HKIR donor cells. However, evaluation of the frequency of E4$^+$ B cells per GC indicated a lower average frequency of such cells in HKIR→B6 as compared with HKI65→B6 chimeras (data not shown). To more quantitatively evaluate this phenomenon, the experiments were conducted using HKI65/Vk10 and HKR/Vk10 (CD45.2) donor cells injected into B6.CD45.1 congenic recipients. The use of this approach allowed us to investigate whether the reduced numbers of canonical GC B cells in HKIR→B6 chimeric mice might be due to inefficient detection of

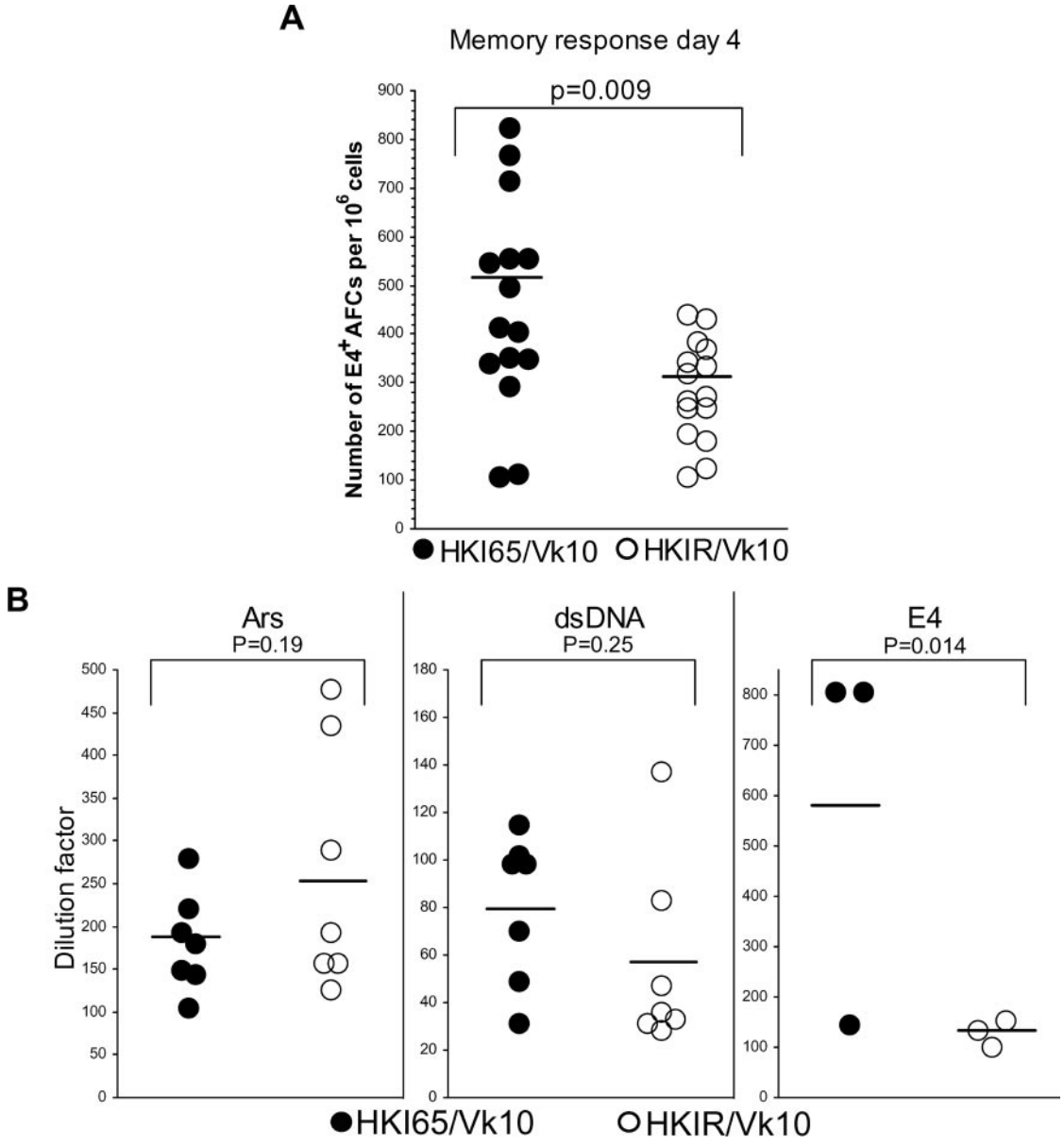

FIGURE 8. The anamnestic AFC response of HKIR/Vk10 B cells is compromised. *A*, HKI65/Vk10 and HKIR/Vk10 chimeric mice were created according to protocol A, boosted with 50 μg of Ars-KLH in PBS 2 mo later and sacrificed 4 days after boosting. The number of $E4^+$ IgG-secreting AFCs was assessed by ELISPOT. Each circle represents the number of AFCs per 10^6 splenocytes obtained from an individual mouse. *B*, Levels of the indicated types of serum Abs were assayed by ELISA in a subset of the mice analyzed in *A*. Data from duplicate assays from individual mice are shown. In both panels, horizontal bars indicate the mean values of the data set, and statistical significance was assessed by the Student *t* test. Roughly 20% of analyzed chimeric mice of both types did not produce a measurable Ig titer in the anamnestic response and data from these mice were not included in the analysis.

these cells due to further down-regulation of their already low BCR levels. Such down-regulation appears to be a general characteristic of proliferative B cells in the GC (37).

Fig. 6*A* shows examples of GCs containing numerous $E4^+$ (and $CD45.2^+$) B cells in both types of HKI/Vk10→B6.CD45.1 chimeric mice. Note the low level of E4 staining of HKI65/Vk10 GC B cells, and the nearly undetectable level of such staining on HKIR/Vk10 GC B cells. Flow cytometric analysis of the frequency of $E4^+$ and $CD45.2^+$ cells at day 5 after immunization that stained with PNA in both types of chimeric mice revealed that, as observed in the two types of HKI→B6 chimeric mice, fewer such cells were present in HKIR/Vk10→B6.CD45.1 chimeras as compared with HKI65/Vk10→CD45.1 chimeras. However, this difference did not rise to the level of statistical significance in the case of CD45.2 staining (Fig. 6*B*).

To more directly analyze the degree of participation in the GC response of canonical HKIR and HKI65 B cells, a quantitative histological approach was taken. Spleen sections were generated from mice 5 days after injection of donor B cells (protocol A), stained with PNA and anti-CD45.2, and images of randomly selected GCs were captured. The numbers of $CD45.2^+$ cells in 5–15 randomly chosen GCs from each mouse were then counted. Fig. 6*C* shows that the average number of $CD45.2^+$ B cells per GC was reduced in HKIR/Vk10→B6 chimeras at this time point by ~30% as compared with HKI65/Vk10→B6 chimeras. Interestingly, this difference appeared to be largely accounted for by an almost complete lack of GCs in sections derived from HKIR/Vk10→B6 chimeras containing >100 $CD45.2^+$ cells.

Analysis of HKIR/Vk10 B cell participation in the GC response at a later time point, using this same protocol, revealed a more

Table I. *Summary of analysis of somatic hypermutations in HKI65 and HKIR knockin loci recovered from microdissected GCs and AFC foci*

	Outside	HKI65	HKIR	HKI65	HKIR
	1′ GCs	1′ GCs	1′ GCs	2′ AFCs[a]	2′ AFCs[b]
Total point mutations	9	57	130	28	243
Number of sequences	4	16	45	2	20
Number of mutated sequences	4	14	40	2	20
Mutation frequency[c] (%)	0.3	0.74	0.59	1.87	2.49
Hot spot mutations[d]	0	21	27	7	55
G+C mutations[e]	5 (55%)	24 (42%)	74 (57%)	16 (57%)	122 (50%)
Sequence length	1,948	7,792	21,915	1,496	9,740

[a,b] Many mutations are shared by different clones. Only data from V regions containing unique point mutations are included.
[c] Mutation frequency is calculated as a total number of mutations divided by total length of sequences analyzed times 100.
[d] Hot spot motifs are RGYW or WRCY with the hot spot underlined.
[e] Number of mutation at G and C positions and their percentage of total mutation is indicated.

striking defect. Fig. 7*A* shows that 8 days after cell transfer the average number of donor B cells per histologically detected GC was reduced ~2-fold ($p < 0.001$) in HKIR/Vk10→B6 as compared with HKI65/Vk10→B6 chimeras. This result was corroborated by flow cytometric analysis of PNA^+ donor B cells ($p = 0.002$, Fig. 7*B*). In addition, further flow cytometric analysis showed that levels of PNA^+, apoptotic donor cells (Annexin V^+) were increased at day 8 in HKIR/Vk10→B6 as compared with HKI65/Vk10→B6 chimeras (Fig. 7*C*), although this difference did not quite reach statistical significance.

Reduced anamnestic response from HKIR/Vk10 B cells

We have previously obtained data indicating that endogenous canonical R55 clonotypes do not efficiently participate in the anamnestic anti-Ars AFC response (19). The above data indicated that this might also be the case for HKIR/Vk10 B cells in chimeric mice. To evaluate this possibility, HKI65/Vk10→B6 and HKIR/Vk10→B6 chimeric mice were generated according to protocol B, and then rested for 6–8 wk. The mice were then boosted with Ars-KLH in PBS i.p. and numbers of splenic $E4^+$, IgG-producing AFCs enumerated by ELISPOT analysis. Fig. 8*A* shows that, in agreement with our previous data, levels of such AFCs were substantially lower in HKIR/Vk10→B6 as compared with HKI65/Vk10→B6 chimeras ($p = 0.009$). Consistent with these results, analysis of serum Ab levels at this same time point showed that while both types of chimera produced similar levels of total anti-Ars Abs, the average titers of $E4^+$ Abs were much higher in HKI65/Vk10→B6 as compared with HKIR/Vk10→B6 chimeras (Fig. 8*B*). Interestingly, and in contrast to the data we obtained in the primary response (Fig. 5*D*), levels of anti-DNA Abs were similar in both types of chimera (Fig. 8*B*), suggesting that HKIR/Vk10 anamnestic AFCs produce canonical Abs with reduced autoreactivity, perhaps due to somatic mutation. We also assayed for the presence of long-lived IgG-producing splenic and BM AFCs via ELISPOT in the above types of chimeras that had not been boosted with Ars-KLH. Somewhat unexpectedly, neither type of chimera gave rise to such AFCs at levels that were significantly above background (data not shown).

Normal frequency, type, and distribution of somatic hypermutation in HKIR GC and post-GC B cells

Reduced participation of HKIR/Vk10 clonotypes in the latter stages of the GC and the anamnestic AFC responses might result from failure to activate V region hypermutation during the GC response, leading to lack of production of substrates for affinity maturation. To address this issue, $E4^+$ GCs (day 5 after cell transfer in protocol (A)), and $E4^+$ AFC foci (day 4 of the anamnestic anti-Ars response using protocol (B)) were microdissected from spleen sections of HKI65→B6 and HKIR→B6 chimeric mice and transgenic canonical V_H genes recovered by PCR and cloned and analyzed by nucleotide sequencing. Table I summarizes the results obtained. Canonical V_H genes recovered from both HKIR and HKI65 $E4^+$ GCs contained similar frequencies of mutation and these mutations were observed to be scattered throughout the length of the genes and were located at similar frequencies in "hot spot" motifs. In addition, canonical V_H genes recovered from anamnestic $E4^+$ AFC foci had a high frequency of mutation that was similar in HKIR→B6 and HKI65→B6 chimeras.

Analysis of the frequency and type of mutations in framework (FR) and CDR regions of these V_H genes was also conducted. Table II illustrates that in both HKI65 and HKIR V_H genes recovered from $E4^+$ GCs, the ratio of mutations causing amino acid replacements was substantially higher than expected of random mutation (2.9) in CDRs, but not in FRs. In anamnestic HKIR AFCs, this ratio in CDRs was somewhat lower than in GCs, but this ratio in FRs was ~1. In addition, in the V_H sequences obtained from anamnestic AFC foci, two mutations in CDR codons 58 and 59 known to increase affinity for Ars (27), were often observed. From the six independent AFC foci analyzed from HKIR→B6 chimeras, five gave rise to multiple V_H sequences that had either one, or both, of these position 58 and 59 mutations (data not shown). These data indicate that phenotypic selection of HKIV

Table II. *R/S (replacement/silent mutation[a]) ratios of Ig V_H regions of the HKI65 and HKIR mice*

	FRs	CDRs	FR1	CDR1	FR2	CDR2	FR3	CDR3	J_H
HKI65[b] primary	25:11	25:3	12:4	6:1	4:3	10:1	9:4	9:1	1:1
HKIR[c] primary	38:12	49:6	19:7	12:1	7:0	28:3	12:5	9:2	3:1
HKIR[d] secondary	24:19	19:8	11:10	3:1	1:1	15:5	12:8	1:2	3:1

[a] Only data from V regions containing unique point mutations are included.
[b,c] Data obtained from primary GC at day 5 postimmunization (protocol A).
[d] Data obtained from secondary AFC foci at day 4 after boosting. Mutations shared by sequences derived from the same AFC focus were counted once.

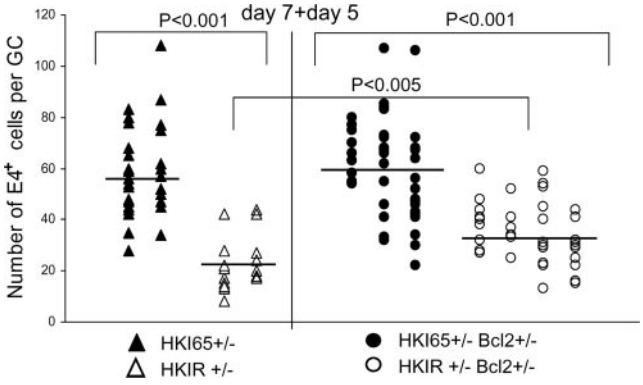

FIGURE 9. Enforced expression of Bcl-2 partially rescues the GC response of HKIR/Vk10 B cells. HKI65/Bcl-2, HKI65, HKIR/Bcl-2, and HKIR chimeric mice were created according to protocol A as described in *Materials and Methods*. Mice were sacrificed on day 5 after adoptive transfer. Spleen cryosections were stained with PNA and E4 and the number of $E4^+$ cells per $E4^+$ GC was counted. At least 10 randomly selected GCs of medium size (20–30 cells in diameter) from each mouse were analyzed. ● and ○, The number of $E4^+$ GC cells per GC derived from HKI65/Bcl2 or HKIR/Bcl2 chimeric mice, respectively. ▲ and △, Analogous data obtained from HKI65 and HKIR single transgenic mice, respectively. Each circle or triangle represents results from an individual GC and each group of circles or triangles represent data from an individual mouse. Horizontal bars represent mean values for each data set. Statistical significance was determined using the Student *t* test.

regions had taken place during the immune response in both types of chimeric mice.

Enforced expression of Bcl-2 partially rescues the GC response of canonical HKIR clonotypes

We previously reported that enforced expression of Bcl-2 in the B cell compartment augments the entry of canonical R55 clonotypes with reduced autoreactivity due to somatic mutation into the Ars-induced memory B cell compartment (19). This suggested that such expression might enhance the participation of canonical HKIR clonotypes in the GC response. To test this idea, HKI65 and HKIR mice were crossed to mice with enforced, B cell-specific expression of Bcl-2 from a transgene and splenic B cells from the resulting double transgenic mice transferred to Ars-KLH-immunized syngeneic mice following protocol A. Splenic B cells from HKIR and HKI65 single transgenic mice were used as controls. Five days after transfer, quantitative histological analysis of the number of $E4^+$ cells per splenic GC were performed. Fig. 9 illustrates that while enforced Bcl-2 expression had little if any effect on the frequency of canonical HKI65 GC B cells, such expression increased the number of HKIR GC B cells by ~50% on average ($p < 0.005$). However, enforced Bcl-2 expression did not elevate the number of $E4^+$ GC B cells in HKIR chimeric mice to levels observed in HKI65 chimeric mice.

Discussion

We recently reported the functional capabilities of canonical HKIR and HKI65 B cells in vitro (22). Although anti-IgM stimulation led to quantitatively reduced proximal BCR-signaling events in canonical HKIR B cells, these cells underwent similar levels of proliferation, class switching, differentiation to AFC phenotype, and induction of activation and costimulatory molecules when stimulated with LPS or anti-IgM plus anti-CD40 and IL-4. Moreover, these B cells presented Ag taken up nonspecifically to T cells as efficiently as canonical HKI65 B cells, but in some assays they presented Ag taken up via the BCR less efficiently than canonical HKI65 B cells. These results indicated that while canonical HKIR clonotypes are not intrinsically anergic, at least by conventional standards, their ability to respond in vivo to foreign Ag might be limited by their low BCR levels. Also, these experiments could not rule out an intrinsic form of anergy that required factors not present in in vitro culture conditions. For example, if cognate autoantigen(s) was not present, or present at a low concentration in the in vitro cultures, this could have resulted in "reversal" of a nonresponsive or partially responsive state maintained in vivo by persistent autoantigen engagement (38).

In this study, by directly comparing the activity of canonical HKIR and HKI65 B cells in vivo after immunization with a TD form of Ars, we show that canonical HKIR clonotypes mount an early primary response comparable to canonical clonotypes lacking the R55 mutation. This included normal homing to follicles, migration to the T-B interface, and induction of costimulatory molecules, proliferation rate, differentiation to primary AFCs, and H chain class switching. In addition, canonical HKIR clonotypes enter GCs and undergo extensive proliferation and somatic hypermutation. These observations are consistent with our previous conclusion, based on the in vitro data summarized above, that canonical HKIR clonotypes are not anergic. In addition, these data demonstrate that the substantially decreased levels of BCR expressed by these clonotypes do not hinder their recruitment, and initial participation in a TD immune response, at least under the experimental conditions used. Interestingly, however, canonical HKIR clonotypes gave rise to an early GC response that was somewhat less robust, and late GC, and anamnestic AFC responses that were substantially reduced, as compared with canonical clonotypes lacking the R55 mutation.

Although the mechanism(s) resulting in reduced participation of canonical HKIR clonotypes in the GC, and subsequent anamnestic AFC response remains to be elucidated, our current data strongly indicate that the mitochondrial pathway of programmed cell death is involved. We previously suggested that during the GC response, canonical clonotypes that had acquired, or failed to lose their preexisting autoreactivity for nuclear autoantigens via hypermutation, were deleted via an apoptotic mechanism (19, 39). This notion was supported by our finding that hybridoma panels generated from the anamnestic anti-Ars responses of mice with endogenous canonical R55 clonotypes only rarely contained cell lines expressing canonical Abs with the R55 mutation. In contrast, introduction of the Bcl-2 transgene into these mice resulted in a dramatically increased yield of such hybridomas. Interestingly, however, analysis of the mAbs produced by these hybridomas revealed lower levels of ANA autoreactivity as compared with the "parent" V region containing only the R55 mutation (19). These results suggested that enforced Bcl-2 expression is only capably of rescuing canonical R55 GC B cells whose autoreactivity has been reduced via somatic hypermutation. Our observation that Bcl-2 only partially restores the GC response of canonical HKIR clonotypes is consistent with this logic, but analysis of the specificity of the BCRs expressed by GC B cells and anamnestic AFCs in Ars-immunized HKIR.Bcl-2 chimeric mice will be required to rigorously test this idea.

The suggestion that a peripheral B cell tolerance checkpoint is operative in the GC has also emerged from the studies of others (1, 40–44), but these studies have as yet failed to provide insight into its mechanism of action. Nonetheless, the data we and others have obtained are consistent with the original "signal one-signal two" hypothesis of Bretscher and Cohn (45). In the case of mature FO B cells, Ag, or autoantigen engagement of the BCR (signal one) might induce abortive activation and entry into GCs, but extended

periods of proliferation, and efficient class switching and differentiation to the memory or long-lived AFC stage would require sustained costimulatory induction (signal two) of B cell growth and survival factors. In TD B cell responses, the latter signals are probably predominantly provided by Th cells, but TLRs and other molecules capable of inducing signaling pathways involved in B cell growth and survival undoubtedly contribute as well. We and others have shown that GCs can be transiently induced in the apparent absence of T cell help (46, 47), and very small numbers of T cells of various subtypes are sufficient to drive efficient GC, but not anamnestic AFC development (48, 49).

Taken together, these considerations suggest that high levels of self or cross-reactive foreign Ag could stimulate entry of autoreactive FO B cells into the GC response, and this would be facilitated when the general level of B cell costimulation was abnormally high, perhaps due to adjuvant effects. Moreover, even anergic B cells can be "rescued" from their nonresponsive and often short-lived state and induced to proliferate, differentiate to AFC phenotype, and enter GCs if robust levels of cognate T cell help is provided to these B cells (50, 51). However, the sustained receipt of costimulation by autoreactive B cells would normally be restricted to situations where autoreactive T cell help (52), or cross-reactive stimulation by foreign Ag, or both, were readily and chronically available. Because canonical HKIR B cells enter the GC reaction with very low levels of BCR, levels that appear to be further reduced during the GC reaction, it is tempting to speculate that as foreign Ag concentrations drop, these B cells are incapable of engaging, internalizing, and presenting sufficient amounts of this Ag to Th cells, resulting in reduced receipt of costimulatory signals. To test these ideas, we are currently determining whether genetic lesions known to alter T cell tolerance pathways influence the GC response and formation of the memory and long-lived AFC compartments by canonical R55 B cells and, if so, whether repeated immunization with Ars exacerbates these effects. Finally, the fact that canonical HKIR clonotypes give rise to a measurable anti-Ars anamnestic AFC response indicates that either the putative GC tolerance checkpoint is not completely efficient, or that the post-GC B cells that give rise to this AFC response have lost autoreactivity due to somatic hypermutation. Experiments are underway to test these ideas.

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Disclosures

The authors have no financial conflict of interest.

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