The Lupus Susceptibility Locus Sle1 Breaches Peripheral B Cell Tolerance at the Antibody-Forming Cell and Germinal Center Checkpoints

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The Lupus Susceptibility Locus Sle1 Breaches Peripheral B Cell Tolerance at the Antibody-Forming Cell and Germinal Center Checkpoints

Raja Vuyyuru, † Chandra Mohan, ‡ Tim Manser, † and Ziaur S. M. Rahman 2*†

We have described a line of V\textsubscript{H} knock-in mice termed HKIR in which the transgenic Igh locus partially encodes “dual-reactive” antichromatin and anti-p-azophenylarsonate (Ars) BCRs. HKIR B cells termed canonical, expressing a particular V\textsubscript{κ}L chain, evade central tolerance by down-regulating BCR levels. Canonical HKIR B cells can be recruited into the primary germinal center (GC) and Ab-forming cell (AFC) compartments via Ars immunization. However, their participation in the GC response rapidly wanes and they do not efficiently contribute to the memory compartment, indicating that they are regulated by a GC tolerance checkpoint. We analyzed the influence of the Sle1 genetic interval, shown to break tolerance of chromatin-reactive B cells, on the behavior of HKIR B cells during the anti-Ars response. Canonical B cells from congenic HKIR.Sle1 mice gave rise to elevated short and long-lived AFC responses, and the attenuated GC and memory responses characteristic of these B cells were relieved in adoptive, wild-type recipients. HKIR GC B cells containing Sle1 expressed increased levels of Bcl-2 and c-FLIP and decreased levels of Fas RNA compared with HKIR controls, suggesting direct alteration of the regulation of the GC response by Sle1. High titers of canonical and anti-dsDNA Abs spontaneously developed in many aged HKIR.Sle1 mice. Together, these data indicate that Sle1 perturbs the action of peripheral tolerance checkpoints operative on autonuclear Ag B cells in both the AFC and GC pathways in a cell autonomous fashion. The Journal of Immunology, 2009, 183: 5716–5727.

During their development, B cells expressing BCRs reactive to autoantigens may be deleted in the bone marrow by apoptosis (1, 2), undergo receptor editing (3, 4), or become anergic (5–7) processes that play key roles in tolerance. However, not all autoreactive B cells are eliminated by these central tolerance mechanisms, as the mature peripheral B cell pool contains multi-reactive B cells that are cross-reactive with autoantigens (8, 9). B cells with BCRs that are “dual reactive” for both autoantigens and foreign Ags could be recruited into a foreign Ag-driven immune response. In addition, the V regions of Ig genes undergo somatic hypermutation in germinal centers (GCs) during T-dependent (TD) immune responses, resulting in de novo generation of autoreactive B cells (10, 11). Therefore, it has been postulated that tolerance mechanisms must operate during primary peripheral B cell development and B cell immune responses (6, 12–13). Studies of autoreactive BCR-transgenic mouse models (14–18) and in humans (19–21) have supported the existence of such peripheral tolerance mechanisms, but these are as yet not well defined.

Systemic lupus erythematosus is a complex autoimmune disease characterized by the production of anti-nuclear Abs (ANAs) affecting multiple organs. The NZB/NZW-derived NZM2410 mouse strain develops a disease that resembles human systemic lupus erythematosus. Genetic linkage studies on this strain have indicated that lupus pathogenesis is a multistep and multifactorial process. Three major genomic intervals for autoimmune susceptibility (Sle1, Sle2, and Sle3/Sle5) were identified in the NZM2410 strain (22). C57BL/6 (B6) mice congenic for each of these loci exhibit different component phenotypes (23–25). For instance, B6.Sle1 mice spontaneously develop high titers of ANAs but these can mediate high penetration of severe glomerulonephritis only in combination with other susceptibility loci (Sle2, Sle3/Sle5, and Yaa or lpr) (26). Congenic recombination of the Sle1 locus has resulted in three subloci named Sle1a, Sle1b, and Sle1c (27). The presence of each of these subloci alone in B6 mice results in only partial autoimmune phenotypes, with the Sle1b subregion appearing to be primarily responsible for loss of B cell tolerance to nuclear autoantigens (28).

We have used an Ig V\textsubscript{H} knock-in line termed HKIR (29, 30) that generates DNA and chromatin-reactive B cells to study peripheral B cell tolerance checkpoinst. The HKIR V\textsubscript{H} transgene, in combination with a single endogenous κ L chain gene, encodes BCRs with specificity for both the hapten p-azophenylarsonate (Ars) and nuclear autoantigens. We term these dual-reactive B cells canonical. Whereas ANA B cells in other BCR-transgenic models such as 3H9 (anti-chromatin) and 2–12H (anti-Smith/ssDNA) undergo receptor editing or anergy (3, 31), HKIR B cells escape these fates by down-regulating their BCRs, resulting in reduced avidity for
nuclear autoantigens (29, 30). These B cells develop to mature follicular phenotype (13, 29, 30) and stably reside in the follicles of peripheral lymphoid organs. Therefore, the HKIR model allows us to study the role and mechanisms of peripheral tolerance checkpoints in regulation of ANA B cell activity.

Due to their dual reactivity, canonical HKIR B cells can be recruited into the GC and Ab-forming cell (AFC) responses via immunization with Ars conjugated to foreign Ag. However, canonical HKIR B cells participate in the early but not the late GC response and do not efficiently seed the memory B cell compartment, suggesting that these cells are regulated by GC/memory tolerance checkpoints (12, 13). To investigate the factors operative in these checkpoints, we previously evaluated the influence of intrinsic deficiencies of the inhibitory Fc receptor FcγRIIB and the Fas death receptor on canonical HKIR B cell participation in the GC/memory B cell pathway (13, 32). The FcγRIIB deficiency increased the participation of canonical HKIR B cells in the primary AFC response, but neither deficiency augmented the participation of these B cells in the late GC or memory responses.

We also previously showed that in B6 mice congenic for the Sle1 genomic interval, GC B cells fail to up-regulate the expression of FcγRIIB, as takes place in nonautoimmune-prone strains of mice (33). In subsequent studies, we demonstrated that this failed up-regulation mapped to a small subinterval of the Sle1 locus containing the NZW allele of the FcγRIIB gene (34). B6 mice congenic for an Sle1 subinterval including this NZW FcγRIIB allele and much of the Sle1a subinterval, but lacking any contribution from the Sle1b subinterval, displayed enhanced primary AFC responses, thus phenocopying B6.FcγRIIB-, and HKIR.FcγRIIB-deficient B cells in this regard (13). Given these results, we wished to determine whether the presence of the NZW FcγRIIB allele in canonical HKIR B cells increased their participation in the late GC and memory B cell responses. However, given our past data showing that a complete FcγRIIB deficiency did not result in such rescue (13), combined with previous results showing that the Sle1b subinterval appears primarily responsible for loss of tolerance to nuclear autoantigens (29, 30), we elected to first evaluate the influence of Sle1 congenic mice on a B6 background and analyzed the primary development and Ars-keyhole limpet hemocyanin (KLH)-driven immune response of their B cells. No major influence of Sle1 on the primary development of these B cells was detected. However, when transferred into syngeneic normal mice, Sle1-bearing canonical HKIR B cells gave rise to enhanced primary anti-Ars AFC responses. More interestingly, the attenuated late anti-Ars GC and memory responses characteristic of HKIR B cells were reversed when these B cells contained Sle1. In addition, we observed high serum titers of spontaneously produced anti- dsDNA and canonical Abs in aged HKIR mice bearing Sle1. Taken together, our results indicate that the presence of the Sle1 interval perturbs both AFC and GC/memory tolerance checkpoints normally operative on ANA B cells and that these alterations function in a cell autonomous fashion. These data are the first to show that a lupus susceptibility locus can alter GC/memory tolerance checkpoints in this manner.

Materials and Methods

Mice

C57BL/6 (B6) and C57BL/6.Sle1+/−Pepc−/BoyJ (B6.CD45.1) mice were purchased from The Jackson Laboratory and then bred in-house. The Ig Vμ knock-in mouse lines HK165 and HKIR were described previously (29, 30). B6.Sle1− mice were also described previously (35). All mice were maintained in a pathogen-free barrier facility, were given only autoclaved food and water, and were 7–9 wk old when used in all experiments except the aging studies. These studies have been reviewed and approved by an appropriate institutional review committee.

Adoptive transfer and immunizations

Two adoptive transfer protocols were used. In one protocol, B6.CD45.1 B6.Sle1+/− or B6 recipient mice were immunized i.p. with 10 μg of Ars-KLH (in alum) 1 wk before transfer (i.v., in PBS) of splenic B cells from transgenic donor mice. Chimeric mice were then injected i.p. with 50 μg of Ars-KLH in PBS immediately after cell transfer. In another protocol, recipient mice were immunized with 100 μg of Ars-KLH in alum i.p. 12–24 h after transfer of splenic B cells from transgenic mice. For secondary immune responses, mice were boosted 6–8 wk after cell transfer and primary immunization following the second protocol. In all experiments, donor B cells were MACS purified using either the E4 mAb, specific for canonical BCRs, or an anti-B220 mAb.

Abs and other reagents

Abs and other reagents used for flow cytometry and immunohistostaining included: FITC-GL7; PE, FITC, and PE-Texas Red for anti-CD80 (16-10A1); FITC-anti-CD86 (GL-1; BD Pharmingen); FITC-metallophilic macrophage 1 (MOMA-1; Serotec); biotin-anti-IgD (11-26; Southern Biotechnology Associates); PE and biotin-anti-mouse CD45.2 (104; eBioscience); SA-PE; SA-Alexa Fluor 633 (Molecular Probes); FITC-peanut lectin (agglutinin) (PNA); and FITC-donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories) and a biotinylated form of the anti-idiotypic mAb E4 (prepared in-house).

Immunohistology

Spleen cryostat sections (5–6 μm) were prepared as described previously (36). Immunohistostaining was performed using the Abs listed above and the stained sections were analyzed using a fluorescence microscope (Leica Microsystems) and images were captured as previously described (37).

Flow cytometry

Three- and four-color flow cytometric analysis was done on cell suspensions prepared from spleens of naive and immunized mice stained with multiple combinations of the Abs listed above. Biotinylated Abs were detected with SA-Chrome. Stained cells were analyzed using a Coulter Epics XL/MCL analyzer. Data were analyzed using FlowJo software (Tree Star).

ELISPOT assays for primary and secondary AFCs

Spleenocytes from chimeric mice were plated at 1 × 10⁶ cells/well and diluted serially (1/2) in multiscreen 96-well filtration plates (Millipore) coated with goat anti-mouse IgM (μ specific) or goat anti-mouse IgG (γ specific; Caltag Laboratories) for 6 h at 37°C. E4′ specific IgM and IgG Abs produced by AFCs were detected using biotinylated anti-clonotypic mAb E4 prepared in-house and SA-alkaline phosphatase (Vector Laboratories). Plates were developed using a Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). ELISPOTs were counted using a computerized imaging video system (Cellular Technology).

ELISA

Anti-Ars, anti-DNA, and clonotype-specific (E4) serum Abs were measured in sera from naive and immunized mice by solid-phase ELISA on 96-well plates (Immulon-4; Thermo Electron) as previously described (10).

GC B cell sorting, DNA extraction, and somatic mutation analysis

B220+, E4+, and PNA+ GC B cells were purified on day 5 of the donor B cell response using a MoFlo activated higher-speed sorter (DakoCytomation). Genomic DNA was prepared from these cells using the DNeasy tissue kit (Qiagen) according to the manufacturer’s instructions. PCR amplification of Vμ transgenes (Tgs), PCR product purification, cloning, sequencing, and mutation analyses were done as previously described (13).

GC B cell sorting, RNA extraction, and real-time RT-PCR

B220+, E4+, and PNA+ GC B cells were purified on day 5 of the donor B cell response using a MoFlo FACS as described above. RNA from LPS-stimulated E4+ canonical B cells were used as control. RNA purification,
reverse transcription of RNA, real time RT-PCR, and generating raw relative quantification (RQ) values for gene expression were performed as described elsewhere (13).

Results

Primary development of HKIR B cells is not altered by the Sle1 locus

We generated HKI65.Sle1 and HKIR.Sle1 mice by crossing B6.Sle1-congenic mice with the HKI65 and HKIR lines (backcrossed to the B6 background for at least 20 generations), respectively. The HKI65 Igh knock-in locus is identical to the HKIR Igh knock-in locus with the exception of a single amino acid codon difference at position 55 in the CDR2 region of the VH gene. In the HKIR locus, this codon specifies arginine (R) and in the HKI65 locus it encodes asparagine (N). The position 55R substantially increases the reactivity of canonical Abs to chromatin and dsDNA, making canonical HKIR B cells far more autoreactive than their HKI65 counterparts (38). However, both types of canonical Abs have similar affinity for Ars and both can be specifically detected using an anti-idiotypic Ab termed E4 (29, 30, 38).

We first evaluated whether bone marrow (BM) development of B cells from both HKI65 (left panels, Fig. 1A) and HKIR (right panels, Fig. 1A) mice was altered in the presence of Sle1. We found no major differences in the percentage of B220<sup>+</sup>IgM<sup>dim</sup> pre-pro B, B220<sup>+</sup>IgM<sup>high</sup> immature and B220<sup>+</sup>IgM<sup>low</sup> mature B cells are shown in three separate gates. B. The percentage of canonical IgM<sup>+</sup>E4<sup>+</sup> cells in the spleens of mice of the indicated genotypes as determined by flow cytometry is shown in rectangular gates. C. Results of flow cytometric analysis of total (B220<sup>+</sup>) and canonical (E4<sup>+</sup>) B cells for surface IgM and IgD (upper and middle rows) and CD21/35 and CD23 levels (lower row). The gates encompass FO B cells (left) and transitional and MZ B cells (right). D. Spleen sections obtained from naive mice of the indicated genotypes were stained with MOMA-1 (green), anti-TCR-<beta> (red), and E4 (blue) and images were captured by fluorescence microscopy. Original magnification of images was ×200. E. Levels of activation markers CD69, CD80, and CD86 were examined by flow cytometry on B220<sup>+</sup>E4<sup>+</sup> B cells from naive HKIR (blue line) and HKIR.Sle1 (red line) mice (upper row). All data are representative of those obtained from three independent experiments.

FIGURE 1. Primary development of HKIR and HKI65 B cells in the presence or absence of Sle1. A. Flow cytometric analysis was performed on BM cells obtained from HKI65.Sle1 and HKIR.Sle1 and HKI65 and HKIR controls after staining with B220 and anti-IgM. The percentage of B220<sup>+</sup>IgM<sup>dim</sup> pre-pro B, B220<sup>+</sup>IgM<sup>high</sup> immature and B220<sup>+</sup>IgM<sup>low</sup> mature B cells are shown in three separate gates. B. The percentage of canonical IgM<sup>+</sup>E4<sup>+</sup> cells in the spleens of mice of the indicated genotypes as determined by flow cytometry is shown in rectangular gates. C. Results of flow cytometric analysis of total (B220<sup>+</sup>) and canonical (E4<sup>+</sup>) B cells for surface IgM and IgD (upper and middle rows) and CD21/35 and CD23 levels (lower row). The gates encompass FO B cells (left) and transitional and MZ B cells (right). D. Spleen sections obtained from naive mice of the indicated genotypes were stained with MOMA-1 (green), anti-TCR-<beta> (red), and E4 (blue) and images were captured by fluorescence microscopy. Original magnification of images was ×200. E. Levels of activation markers CD69, CD80, and CD86 were examined by flow cytometry on B220<sup>+</sup>E4<sup>+</sup> B cells from naive HKIR (blue line) and HKIR.Sle1 (red line) mice (upper row). All data are representative of those obtained from three independent experiments.

Both HKI65 and HKIR BCRs promote efficient development of canonical B cell precursors to mature follicular (FO) but not to marginal zone (MZ) B cells (13). As shown in Table I and Fig. 1B, the total number of B220<sup>+</sup> cells and the total number and frequency of canonical E4<sup>+</sup> cells in HKI65.Sle1 and HKIR.Sle1 were comparable to those of HKI65 and HKIR mice. In addition, nearly all E4<sup>+</sup> cells (99%) from mice of all four genotypes were IgM low IgD<sup>+</sup> (Fig. 1C, second row) and CD21<sup>+</sup>CD23<sup>+</sup> (Fig. 1C, third row) FO B cells. The profile of bulk B220<sup>+</sup>IgM<sup>+</sup>IgD<sup>+</sup> FO B cells containing transitional and MZ B cells and IgM<sup>+</sup>IgD<sup>+</sup> FO B cells in HKI65.Sle1 and HKIR.Sle1 mice was similar to that of HKI65 and HKIR controls, respectively (Fig. 1C, first row). The lower percentage of IgM<sup>+</sup>IgD<sup>+</sup> T plus MZ and slightly higher percentage of IgM<sup>+</sup>IgD<sup>+</sup> FO B cells observed in HKIR and HKIR.Sle1 mice resulted from the more pronounced reduction of the MZ B population in HKIR mice, as described previously (13). We also observed substantially lower levels of surface BCR (both IgM and IgD) on canonical HKIR and HKIR.Sle1 E4<sup>+</sup> B cells compared with their HKI65 counterparts (Fig. 1C, second row), indicating that the adaptive central tolerance pathway that results...
in reduced avidity of canonical HKIR B cells for autoantigen was not influenced by Sle1.

We next performed an immunohistological analysis of spleen sections obtained from naive mice by staining with E4 (blue), anti-TCR-β (red), and MOMA-1 (green) (Fig. 1D). MOMA-1 stains for metallocophilic cells located at the border of follicles and the MZ. E4+ cells were confined mostly to B cell areas in the white pulp in HKI65.Sle1 (Fig. 1D, upper right) and HKIR.Sle1 (Fig. 1D, lower right) mice with few E4+ cells in the T cell and MZ areas, similar to that observed in HKI65 (Fig. 1D, upper left) and HKIR (Fig. 1D, lower left) controls.

We also examined the surface levels of the activation/costimulatory markers CD69, CD80, and CD86 to determine whether HKIR B cells were preactivated in the presence of Sle1. The levels of these markers on ex vivo HKIR.Sle1 naive E4+ cells (Fig. 1E, red lines, upper row) were comparable to HKIR controls (Fig. 1E, blue lines, upper row).

Enhanced primary (short-lived) AFC responses of canonical HKIR B cells in the presence of Sle1

Due to their dual-reactivity (Ars and ANA), canonical HKIR B cells can be recruited into the GC and AFC responses with Arssel conjugated foreign Ag. By performing adoptive transfer experiments, we previously showed that during the ensuing anti-Ars AFC response, there was no significant difference between canonical HKI65 and HKIR B cell differentiation into IgM- and IgG-producing primary AFCs (12, 13). There are several reasons for using the adoptive transfer approach and not examining the GC and AFC responses in the primary mice. First, the precursor frequency of canonical clonotype B cells in HKI65 and HKIR primary mice is very high (approximately a million E4+ Ars+ B cells in the spleen). Apparently as a consequence, even high doses of Ars Ag do not elicit normal levels of AFCs or GCs and these mice fail to develop memory. In our transfer experiments, we injected 2 × 10^6 total purified B cells containing at the most 1 × 10^5 E4+ donor B cells. Second, adoptive transfer allows us to study B cell-intrinsic effects of Sle1, e.g., by transferring B cells expressing Sle1 into wild-type recipients. Third, using allotype markers, we can distinguish responding donor cells from recipient cells and this allows the recipient cells to be used as “internal controls” (e.g., for the magnitude of the overall GC response).

Two × 10^6 B cells (containing ~10^5 E4+ cells) from HKIR.Sle1 and HKIR control mice were adoptively transferred to syngeneic B6 recipients. B6 mice lack the V_H gene necessary to encode canonical E4+ Abs (39). We then immunized the resulting chimeric mice with Ars-KLH 12–24 h later and donor cell-derived E4+ AFCs were quantified by ELISPOT assay on day 6 after immunization. The numbers of IgM producing E4+ AFCs in mice receiving HKIR.Sle1 B cells were significantly higher compared with those of HKIR controls (Fig. 2A). In addition, IgG-producing E4+ AFCs (Fig. 2B) in mice receiving HKIR.Sle1 B cells were almost 2-fold more frequent on average and ~30% of these mice had significantly higher numbers of E4+ AFCs compared with controls. As discussed in more detail below, this variability might be explained by the incomplete penetrance of the Sle1 locus on the AFC phenotype in B cells derived from young mice.

Reduced participation of HKIR E4+ autoreactive B cells in the foreign Ag-driven GC and memory responses

We previously have shown reduced participation of HKIR E4+ compared with HKI65 E4+ B cells in the anti-Ars GC response (13), in which we adoptively transferred E4+ AFCs (Fig. 2B) in mice receiving HKIR.Sle1 B cells were almost 2-fold more frequent on average and ~30% of these mice had significantly higher numbers of E4+ AFCs compared with controls. As discussed in more detail below, this variability might be explained by the incomplete penetrance of the Sle1 locus on the AFC phenotype in B cells derived from young mice.

Table 1. Absolute number of B220+ and E4+ and frequency of E4+ cells in the presence or absence of Sle1

<table>
<thead>
<tr>
<th>Total No. of B Cells a (×10^6)</th>
<th>Total No. of E4+ Cells b (×10^6)</th>
<th>Frequency of E4+ Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knock-in mice c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HKI65</td>
<td>15.0 ± 3.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>HKI65.Sle1</td>
<td>16.5 ± 5.5</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>HKIR</td>
<td>16.7 ± 4.7</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>HKIR.Sle1</td>
<td>15.6 ± 4.3</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

a Total number of B (B220+) cells on lymphocyte gate from three to four mice of each genotype analyzed.

b Total number of E4+ cells out of total lymphocytes.

c HKI65+/− and HKI65+/+ knock-in mice bearing or not bearing Sle1.

FIGURE 2. Primary IgM and IgG AFC responses of HKIR canonical B cells in the presence or absence of Sle1. HKIR and HKIR.Sle1 canonical B cells were transferred to B6 recipients that were subsequently immunized with Ars-KLH (see Materials and Methods). The number of splenic E4+ IgM (A, upper panel) and IgG (B, lower panel) secreting AFCs were measured by ELISPOT assay 6 days after immunization. Each circle represents the number of E4+ AFCs per 1 × 10^6 splenocytes obtained from an individual chimeric mouse. Open and closed circles represent data from HKIR and HKIR.Sle1 chimeric mice, respectively. Horizontal bars represent the average number of E4+ AFCs. Statistical analysis was performed using Student’s t test. The data for IgM-producing AFCs were obtained from three independent experiments and the data for IgM-producing AFCs were obtained from two independent experiments.
transfer of E4+ B cells. This protocol results in a highly synchronized immune response from the input donor B cells and, thus, greatly facilitates analysis of the GC response of canonical E4+ B cells in the chimeric mice.

Consistent with our previously published data where we transferred splenocytes (12, 13), we found similar results with transferring purified E4+ B cells. Fig. 3A illustrates histological data showing that in HKIR→CD45.1 chimeras, CD45.2+ (red) canonical HKIR B cells clearly enter splenic GCs (stained with GL7, green) as evidenced by the presence of CD45.2+ cells within GL7+ B cell foci (yellow overlap staining, Fig. 3A). Therefore, the reduced participation of canonical HKIR B cells in GC and memory responses cannot be attributed to these cells being excluded from GCs, which might be the case for anergic B cells. However, the expansion of these B cells in GCs appeared limited. We observed mostly small CD45.2+ GCs with a reduced number of CD45.2+ cells in HKIR→B6.CD45.1 mice, 10% of CD45.2+ GCs in HKIR→B6.CD45.1 were large (Fig. 4E), indicating enhanced expansion of canonical HKIR GC B cells bearing the Sle1 locus. We did not, however, observe that HKIR B cells formed or participated in extrafollicular GC-like reactions as previously observed in MRL/lpr mice (43).

To determine whether the difference in the GC response between canonical HKIR and HKIR.Sle1 cells might result from a generalized defect in all B cells expressing Sle1, GC responses induced by the TD Ag sheep RBC (SRBC) were evaluated in B6 (blue) and B6.Sle1 (red) mice (Fig. 4F). As described above, splenic GCs in these mice defined histologically were counted and sorted into small, medium, and large categories. This analysis revealed that the number of small, medium, and large GCs in SRBC immunized B6 and B6.Sle1 mice did not significantly

### FIGURE 3. Reduced anti-Ars GC and memory responses of HKIR B cells. A. Spleen sections, obtained from Ars-KLH-preimmunized B6.CD45.1 mice 5 days after transfer of HKI65 or HKIR CD45.2 B cells (see Materials and Methods), were stained with anti-CD45.2 (red) and the GC marker GL7 (green). Two representative GCs from each type of mouse are shown. B. The number of CD45.2+ B cells per GC were counted in eight randomly chosen small- and medium-sized CD45.2+ GCs per mouse. Each circle indicates the number of CD45.2+ cells counted from a particular GC in HKI65→CD45.1 (blue) and HKIR→CD45.1 (red) chimeric mice. C, Donor cell-derived E4+ IgG-secreting secondary AFCs were measured by ELISPOT assay on day 4 after secondary immunization. Chimeric mice were created by transfer of HKIR or HKI65 splenic B cells followed by Ars-KLH immunization. Mice were rested for 8 wk before boosting. Each circle represents the number of E4+ AFCs per 10⁶ splenocytes obtained from a single chimeric mouse. Blue and red circles represent the data from mice receiving HKI65 and HKIR donor cells, respectively. D, Anti-Ars (left two panels) and E4 (right two panels) Ab serum titers were measured by ELISA on day 4 after secondary immunization. The serum samples assayed in D were obtained from the same mice described in C. Horizontal bars represent averages of the data set in B–D. Statistical analysis was performed using Student’s t test.

GCs were categorized into three groups: small (10–25 cell diameters), medium (26–39 diameters), and large (40 or more diameters). Very few large GCs were observed in either type of chimera. The frequency of CD45.2+ GCs in HKIR→B6.CD45.1 chimeras was similar to that observed in HKI65→B6.CD45.1 mice (data not shown), but the number of CD45.2+ cells per GC differed. CD45.2+ cells in randomly chosen small and medium GCs were counted. The number of CD45.2+ cells per HKIR→B6.CD45.1 CD45.2+ GCs was significantly reduced by an average of nearly 2-fold (Fig. 3B) compared with that found in HKI65→B6.CD45.1 CD45.2+ GCs.

To study the differences in anti-Ars memory responses of HKIR vs HKI65 canonical B cells, chimeric mice were rested for 2 mo after transfer of 2 × 10⁶ B cells (containing ~10⁴ E4+ cells) from HKIR or HKI65 mice and immunization with Ars-KLH. IgG-producing E4+ AFCs were quantified by ELISPOT assay on day 4 after secondary immunization with Ars-KLH in saline. The anamnestic response of HKIR E4+ cells (red, Fig. 3C) was 3- to 4-fold lower compared with HKI65 controls (blue, Fig. 3C). In agreement with these AFC data, anti-Ars and E4+ total serum Ig titers in HKIR→B6 mice (red, Fig. 3D) were substantially reduced compared with HKI65→B6 controls (blue, Fig. 3D). Altogether, our previously published data in which we transferred splenocytes (13) are consistent with the current data generated via adoptive transfer of B cells. This allows us to study the B cell-specific effect of Sle1 on the anti-Ars AFC and GC responses by transferring HKIR B cells expressing Sle1.

The GC response of canonical HKIR B cells is augmented in the presence of Sle1

To study the influence of Sle1 on the action of the GC tolerance checkpoint indicated by the data above, 2 × 10⁶ purified canonical HKIR or HKIR.Sle1 B cells (B220+, E4+, and CD45.2+) were transferred to B6.CD45.1 recipients that had been immunized with Ars-KLH 1 wk earlier. Flow cytometry analysis of splenocytes obtained on day 5 of the donor B cell response revealed a significant increase in the percentage of donor-derived CD45.2+ PNA− GC B cells in HKIR.Sle1→B6.CD45.1 mice compared with HKIR→B6.CD45.1 controls (Fig. 4, A and B). These data are consistent with immunohistology results (Fig. 4C) illustrating that more CD45.2+ cells (Fig. 4C, red and yellow overlap staining) per GC were observed in HKIR.Sle1→B6.CD45.1 mice (Fig. 4C, right two panels) compared with HKIR→B6.CD45.1 controls (Fig. 4C, left two panels).

Semiquantitative analysis of CD45.2+ cells in histologically defined GCs, conducted as described above, corroborated these findings. The number of CD45.2+ cells per HKIR.Sle1→B6.CD45.1 GCs was significantly higher compared with that observed in HKIR→B6.CD45.1 GCs (Fig. 4D). In addition, although we found few large CD45.2+ GCs in HKIR→B6.CD45.1 mice, 10% of CD45.2+ GCs in HKIR.Sle1→B6.CD45.1 were large (Fig. 4E), indicating enhanced expansion of canonical HKIR GC B cells bearing the Sle1 locus. We did not, however, observe that HKIR B cells formed or participated in extrafollicular GC-like reactions as previously observed in MRL/lpr mice (43).
differ. Consistent with these data, the average number of secondary 4-hydroxy-3-nitrophenyl acetyl (NP)-specific IgG1 AFCs was also similar in both B6 and B6.Sle1 mice described below (see Fig. 7E) in which mice were immunized with another TD-Ag (NP-chicken γ-globulin (CGG)).

Finally, to test whether the augmented anti-Ars AFC and GC responses of canonical HKIR B cells containing Sle1 could lead to increased titers of serum Abs derived from these cells, we measured the levels of anti-Ars (Fig. 4G) and E4⁺ (Fig. 4H) Abs in the sera of the chimeric mice described in Fig. 4, A–E, and found that
Augmented secondary (long-lived) AFC response of HKIR E4<sup>+</sup> cells in the presence of Sle1

Some AFCs generated during TD immune responses migrate to and reside in the BM where they are relatively long-lived. Therefore, we next examined whether the Sle1 locus led to an increased number of long-lived BM E4<sup>+</sup> AFCs derived from canonical HKIR B cells. For this purpose, 2 × 10<sup>6</sup> B cells (containing ~10<sup>5</sup> E4<sup>+</sup> cells) from HKIR.Sle1 or HKIR control mice were transferred to B6 recipients that were immunized 12 h later with Ars-KLH in alum. Long-lived E4<sup>+</sup> AFCs were quantified in the BM by ELISPOT assay on day 30 after immunization. The numbers of both IgM (Fig. 5A) - and IgG (Fig. 5B) -producing E4<sup>+</sup> BM AFCs in mice receiving HKIR.Sle1 B cells were found to be significantly higher compared with HKIR controls.

The influence of Sle1 in post-GC memory pathways

To evaluate whether Sle1 alters post-GC memory pathways taken by ANA B cells, we transferred purified B cells (2 × 10<sup>6</sup>) from HKI65.Sle1 and HKIR.Sle1 mice and HKI65 and HKIR controls into B6 mice that were immunized with Ars-KLH 12 h later. Chimeric mice were rested for 2 mo and memory responses were evaluated by quantifying secondary E4<sup>+</sup> IgG splenic AFCs in these mice by ELISPOT assay 4 days after boosting with Ars-KLH in saline. In accordance with our previous data (13) and the data shown in Fig. 3C, we found a significant reduction in the number of E4<sup>+</sup> secondary IgG AFCs (Fig. 6, A and B) in HKIR→B6 mice (red circles) compared with HKI65→B6 control (blue circles). Surprisingly, we found a complete absence of E4<sup>+</sup> secondary IgG AFCs in both HKI65.Sle1→B6 (green circles) and HKIR.Sle1→B6 (black circles) mice (Fig. 6, A and B).

To determine whether this was due to a defect in IgH class switching leading to the accumulation of secondary AFCs producing IgM, we quantified IgM-producing E4<sup>+</sup> AFCs and obtained analogous results (data not shown). We also measured serum Ab titers by ELISA and found low to undetectable levels of anti-Ars

30% of HKIR.Sle1→B6.CD45.1 mice had significantly higher anti-Ars and E4<sup>+</sup> titers than those of HKIR→B6.CD45.1 mice, again consistent with an incomplete penetrance of the effect of the Sle1 locus on this response.
we next considered whether the complete absence of E4 NZW origin that encodes at least 100 transcripts. Therefore, the influence of the Sle1 locus on the secondary AFC response.

The Sle1 interval is a 37-centiMorgan long genomic segment of NZW origin that encodes at least 100 transcripts. Therefore, we next considered whether the complete absence of E4+ secondary AFCs in HKI65.Sle1→B6 and HKIR.Sle1→B6 mice might result from rejection of memory B cells bearing Sle1 by the B6 recipient mice.

To test this possibility, we transferred B cells from HKIR.Sle1 mice into B6 and B6.Sle1 mice and quantified E4+ secondary AFC responses as described above. Although we found a complete lack of such responses in HKIR.Sle1→B6 mice, secondary E4+ IgG AFCs in HKIR.Sle1→B6.Sle1+/+ mice were readily observed (data not shown), indicating that HKIR.Sle1 B cells are rejected or suppressed by the B6 hosts due to allotype differences in an Ag(s) encoded in the Sle1 locus.

As such, we next transferred B cells from HKIR and HKIR.Sle1 mice into (B6 × B6.Sle1)F1 (termed B6.Sle1+/−) mice. Previous studies have indicated that the influence of the Sle1 locus on T and accessory cell function is recessive (44, 45). We evaluated memory responses in these chimeric mice by quantifying the numbers of secondary splenic AFCs as described above and found that the number of secondary E4+ IgG AFCs on average was 2-fold higher in HKIR.Sle1→B6.Sle1+/− mice compared with HKIR→B6.Sle1+/− controls (Fig. 7A).
Although the p value between these two groups did not reach statistical significance, 30% of B6.Sle1+/− mice receiving HKIR.Sle1 B cells had a 5- to 6-fold larger number of E4+ IgG AFCs compared with HKIR→B6.Sle1+/− controls. These data are consistent with the primary AFC data shown in Fig. 2 indicating that the influence of the Sle1 locus on the AFC response of HKIR B cells is incompletely penetrant. In addition, anti-Ars (Fig. 7B) and E4+ (Fig. 7C) total serum Ig titers in HKIR→B6.Sle1+/− mice (red) were also significantly higher compared with HKIR→B6.Sle1+/− controls (blue).

Finally, we considered the possibility that allogeneic effects that resulted in rejection or suppression of HKIR.Sle1 memory B cells in B6 hosts might be responsible for the enhanced primary AFC and GC responses of HKIR.Sle1 B cells in HKIR→B6 chimeric mice (Figs. 2, 4, and 5). However, chimeric mice created by transfer of HKIR.Sle1 B cells into B6.Sle1+/− hosts also gave rise to enhanced primary AFC (Fig. 7D) and GC (Fig. 7E) responses. In contrast, the same responses of HKI65 B cells in B6.Sle1+/− hosts were not enhanced by the presence of Sle1 in these donor B cells. To confirm these data, in another set of experiments, we transferred HKIR and HKIR.Sle1 B cells into B6.Sle1+/− hosts and performed similar analysis and obtained analogous results for GC responses (Fig. 7F). Interestingly, we observed significantly increased IgG-producing E4+ AFCs in B6.Sle1+/− mice receiving HKIR.Sle1 B cells compared with HKIR controls (Fig. 7G). In total, these results suggest that allelogeneic differences between HKIR.Sle1 B cells and B6 hosts do not overtly influence the outcome of the primary immune response produced by these B cells.

Sle1 alters expression of genes regulating GC B cell survival but not differentiation

In normal mice, the transcription factors Blimp-1 and Xbp-1 are up-regulated in B cells committed to the AFC pathway and drive development to secretory phenotype. Conversely, most GC B cells express very low levels of these factors but express high levels of the transcription factor Bcl-6, which appears to suppress the expression of Blimp-1 and Xbp-1 (46–49). Elevated levels of Blimp-1 and Xbp-1 (46–49) indicated that the influence of the Sle1 locus could potentially promote the development of these cells to AFCs. To test this idea, 2 × 10^6 B cells from HKIR or HKIR.Sle1 mice were transferred into B6 mice that had been immunized with Ars-KLH 1 wk earlier. Five days after transfer, E4+ GC B cells (B220^highE4^+PNA−) were purified by FACS from both types of chimeric mice and RNA was extracted and used to perform quantitative PCR for levels of Blimp-1, Xbp-1, and Bcl-6 transcripts. Bcl-6 RNA was up-regulated to a similar extent and the levels of Blimp-1 and Xbp-1 RNA were comparably low in both types of GC B cells (Fig. 8A). These data indicate that the enhanced AFC response of canonical HKIR.Sle1 B cells was not due to increased expression of Blimp-1/Xbp-1 by these cells during the GC response.

Next, to examine whether the augmented anti-Ars GC response of HKIR B cells expressing Sle1 resulted from alteration of expression of genes that regulate GC B cell survival, we performed quantitative PCR for Bcl-2, Bcl-xL, c-FLIP, and Fas transcripts in GC B cells in the presence or absence of Sle1 with the same RNA samples used to generate the data shown in Fig. 8A. Interestingly, we found Bcl-2 and c-FLIP transcript levels were up-regulated 4- to 5-fold in HKIR.E4+ GC B cells containing the Sle1 interval compared with FO or HKIR GC B cells without Sle1 (Fig. 8B). Conversely, we observed decreased levels of Fas transcripts in HKIR.Sle1 GC B cells compared with controls (Fig. 8B). We performed similar experiments using RNA samples obtained from HKIR→B6.Sle1+/− and HKIR.Sle1→B6.Sle1+/− GC B cells which revealed analogous results (data not shown).

Equal if not higher frequency of somatic mutations in the V regions of HKIR.Sle1 canonical GC B cells

Next, we determined whether the augmented anti-Ars GC response of canonical B cells correlated with the increased frequency of somatic mutations in the V regions of transgenes in mice receiving HKIR.Sle1 B cells compared with HKIR controls. B220^E4^+PNA− GC B cells were purified by FACS sorter on day 5 of donor B cell responses in preimmunized B6.Sle1+/− recipients. V regions of H chain knock-in transgenes were amplified, cloned, and sequenced as previously described (13). As shown in Table II, the somatic hypermutation frequency in Ig H chain transgenes in GC B cells obtained from HKIR.Sle1→B6.Sle1+/− mice (0.52%) was equal if not higher than that observed in HKIR→B6.Sle1+/− control GCs (0.33%).

*High titers of spontaneously produced canonical and anti-dsDNA Abs in the presence of Sle1*

To test whether data obtained through the adoptive transfer and immunization protocols described were reflective of events that
might take place in HKIR mice that were not overtly immunized, we measured anti-dsDNA, E4, and anti-Ars serum Ab titers by ELISA (10) in naive HKIR mice at 3 and 6 mo of age and found low to undetectable levels of these Abs (C, Fig. 9). In contrast, we observed increased titers of anti-dsDNA, E4, and anti-Ars Abs in most 3- and 6-mo-old HKIR.Sle1 mice (●, Fig. 9), consistent with the data obtained from chimeric mice and indicating that the operation of a peripheral B cell tolerance checkpoint(s) is impaired in unmanipulated HKIR mice in the presence of Sle1.

**Discussion**

Extensive previous studies by Wakeland and colleagues (23, 28, 50) have shown that of the three major NZW-derived loci that contribute to lupus-like disease in mice with a C57BL/6 (B6) background (Sle1, Sle2, and Sle3), only Sle1 appears to cause major intrinsic changes in B cell tolerance and immunoregulatory pathways, particularly to chromatin-based autoantigens. This insight led these investigators to propose an epistatic model for the development of autoimmune disease in this system in which the first step is mediated by genes in the Sle1 interval, resulting in a loss of B cell tolerance. This is followed by secondary events mediated by genes in the Sle2 and Sle3 regions, resulting in loss of T cell tolerance, epitope spreading, and the development of autoantigen-driven production of pathological IgG Abs (51–53). Nonetheless, further genetic dissection of the Sle1 locus revealed three subregions termed Sle1a, Sle1b, and Sle1c that produce different component autoimmune phenotypes (27). Subsequently, it has been shown that the B cell-intrinsic effects conferred by the Sle1 interval mainly map to the Sle1b subregion (28, 54), while the Sle1a and Sle1c regions were reported to be largely influencing CD4 T cell tolerance and regulatory T cell numbers and activity (45, 55). As such, in this study, we chose to perform adoptive transfers of chromatin-Ars dual-reactive, Sle1-bearing HKIR B cells into B6 hosts. There are caveats associated with the use of the preimmunization/ adoptive transfer protocol that need to be taken into account when interpreting the significance of the data to a more general understanding of the role of the GC tolerance checkpoint in the regulation of the development of autoimmunity. These include the preexisting T cell help and GC responses generated by recipient cells and the high precursor frequency of donor cells specific for one Ag. Nonetheless, we have previously shown using this protocol that canonical HKIR65 B cells produce Ars-driven early primary responses that are qualitatively indistinguishable from those produced by canonical B cell clonotypes in normal (i.e., A/J) mice (12). The distinct advantage of using this protocol over and above allowing the participation of ANA B cells in immune responses to be directly evaluated via techniques such as flow cytometry and histology is that we can study how various genes and genetic loci influence the action of B cell tolerance pathways in a B cell autonomic manner. In the case of the Sle1 locus, this is particularly important, since subregions of this locus have been shown to influence the behavior of both T cells and myeloid cells (45, 55).

Our studies were also motivated by our previous findings that a deficiency in FcγRIIB, whose gene maps to a location between the Sle1a and Sle1b subregions, generically perturbed the activity of both strongly and weakly autoreactive B cells in the AFC, but not the GC pathway (13). Moreover, analysis of a newly developed congenic line of mice containing the Sle1-derived FcγRIIB gene, but lacking the other major subregions of the Sle1 locus, demonstrated that B cell tolerance was maintained in these mice, but short-lived AFC responses to foreign Ags were amplified, particularly for IgG production (34). In total, these data suggested that other genes in the Sle1 interval must be responsible for loss of B cell tolerance to chromatin-based autoantigens. Indeed, we have shown that genes of the SLAM/CD2 family present in this interval result in perturbed deletion, receptor editing, and anergy induction in the soluble hen egg lysozyme/anti-hen egg lysozyme Ig-transgenic model of B cell tolerance (28). In particular, these phenotypes correlated with expression of a particular allele of a SLAM/ CD2 gene termed Ly108.1, which appears to attenuate BCR signaling.

Since canonical HKIR B cells evade central and early peripheral tolerance pathways via adaptive down-regulation of BCR levels, the HKIR model afforded us the potential to study the effects of the Sle1 interval on tolerance pathways operative during Ag-driven immune responses and beyond. This potential was fulfilled, as the analysis of the primary development of canonical and other B cells in HKIR.Sle1 mice showed no major differences in BCR downregulation and progression to mature FO phenotype and locale,
strongly suggesting that genes in the Sle1 interval do not perturb the primary adaptive tolerance pathway taken by these B cells. Moreover, these results demonstrate that the previously described alterations in T cell development and activity conferred by the Sle1 interval (45, 54–56) do not indirectly influence the primary development and tolerance of HKIR B cells.

In contrast, analysis of the Ars-driven immune response in chimeric mice created by injection of HKIR.Sle1 B cells into B6 hosts revealed significant perturbation of the activity of these B cells in both the AFC and GC pathways. Importantly, this did not appear to result from a generic alteration of the GC response, since the presence of the Sle1 interval did not change the number and size of the GCs in SRBC-immunized B6.Sle1 mice compared with B6 or the participation of HKI65 B cells in the GC response. Anti-NP secondary responses in B6.Sle1 mice were also similar to B6 controls, suggesting that the GC response induced by this Ag was not quantitatively altered by Sle1.

Given our previous results, the most likely candidate gene that results in the amplified AFC response characteristic of HKIR.Sle1 B cells is the NZW FcγRIIB allele. We and others have shown that this allele fails to be up-regulated on GC B cells (33, 57, 58). Although we have not yet detected any impact of this failed up-regulation on the quantity or quality of the GC response, lower levels of expression of this Fc receptor on B cells in the AFC pathway would be expected to perturb the immune complex-mediated feedback that controls the number and activity of B cells in this pathway (59). Indeed, our previous studies of B6-congenic mice bearing the NZW FcγRIIB allele strongly support this possibility (34). Interestingly, however, only a subset of HKIR.Sle1→B6 chimeric mice displayed increased primary and anamnestic AFC responses, indicating that the penetrance of the NZW FcγRIIB allele on AFC feedback regulation is incomplete and is influenced by stochastic, perhaps environmental factors. This is in contrast to our previous findings on analogous chimeric mice generated using FcγRIIB-deficient HKIR B cells (13), suggesting that the effects of the NZB FcγRIIB allele do not completely phenocopy a FcγRIIB deficiency, perhaps because this allele is indeed expressed, albeit at a lower level than in wild-type mice.

In contrast, the elevated GC and memory responses produced by HKIR.Sle1 compared with HKIR B cells are likely not due to the NZW FcγRIIB allele alone, since we previously showed that a complete FcγRIIB deficiency in HKIR B cells did not influence the substantially reduced participation of these autoreactive B cells in the GC and anamnestic responses (13). Given that the SLAM/CD2 Ly108.1 allele has been shown to attenuate BCR signaling (28), it is possible that reduced signaling through the BCR complex in HKIR.Sle1 GC B cells spares them from the action of an autoantigen-driven GC tolerance checkpoint that normally mediates their deletion. Lack of a direct influence of SLAM alleles on the magnitude of the primary AFC response might be explained by the fact that the BCR is down-regulated on GC B cells, creating a situation where levels of BCR signaling become limiting and placing particular importance on the efficient functioning of signaling components that act downstream of the BCR. Testing the above ideas will require the development of new lines of HKIR-congenic mice containing various subintervals of the Sle1 locus including Sle1b and the region between Sle1a and Sle1b containing the NZW FcγRIIB allele.

These are the first data demonstrating that a lupus susceptibility locus can alter GC tolerance checkpoints in a B cell autonomous fashion. Although the mechanism of perturbation of the GC tolerance checkpoint by Sle1 remains to be clarified, our data suggest that this is taking place via alteration of GC B cell survival. We found that the levels of expression of Blimp-1, Xbp-1, and Bcl-6 genes involved the regulation of B cell differentiation in GC B cells were not altered by Sle1. However, expression of Bcl-2 and c-FLIP RNA was increased and Fas RNA expression was decreased in GC B cells in the presence of Sle1. Although the Bcl-2 and c-FLIP genes are located on the same chromosome (chromosome 1) as Sle1, they are 20 and 40 centiMorgans upstream of the Sle1 locus, respectively. Thus, Bcl-2, c-FLIP, and Fas are likely to act downstream of genes in the Sle1 interval.

Our studies also revealed an unanticipated caveat to the use of congenic mice in adoptive transfer studies for the analysis of the genes that contribute to the development of autoimmunity. Although HKIR.Sle1→B6 chimeras gave rise to primary immune responses that were equivalent or elevated compared with HKIR→B6 mice, the former produced weak to undetectable anamnestic responses. However, when analogous chimeras were generated using Sle1 homozygous or heterozygous recipients, the anamnestic responses produced by HKIR.Sle1 B cells were at least equivalent and often more robust than those developed by HKIR B cells. These results strongly suggest that alloantigens encoded in the Sle1 interval activate recipient T cells, resulting in the eventual killing or suppression of donor B cells by these primed T cells. Clearly, such allogeneic effects could either enhance or inhibit the action of various autoimmunity-influencing gene products in other contexts, confounding interpretation of the role of such gene products in the development of autoimmunity. However, such allogeneic effects appeared to have little or no influence on the primary immune responses of HKI65.Sle1 and HKIR.Sle1 B cells in B6 hosts (Fig. 7).

Finally, our analyses showed that HKIR.Sle1-congenic mice spontaneously develop significant titers of anti-DNA and canonical serum Abs by 3 mo of age, and serum levels of such Abs are further increased at 6 mo of age. This suggests that even in the absence of overt immunization, defects in peripheral tolerance checkpoints due to the presence of Sle1 allow activation and eventual anti-DNA autoantibody production by HKIR B cells. Given the results we obtained from Ars-immunized HKIR.Sle1 B cell chimeric mice, it is tempting to speculate that the production of serum autoantibodies in HKIR.Sle1 mice is driven, at least initially, by environmental Ags cross-reactive with HKIR BCRs. This perspective leads to a new kinetic model for how genetic susceptibility loci like Sle1 may sometimes operate to promote the development of autoimmunity. In this model, the influence of these loci would be most prominent not before, but after an autoreactive B cell was recruited into an immune response.

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