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Ligation of Surface Ig by Gut-Derived Antigen Positively Selects Chicken Bursal and Peripheral B Cells

Dariush Davani,* Zeev Pancer,† and Michael J. H. Ratcliffe*

In many mammals and birds, B cell lymphopoiesis takes place in GALT, such as the avian bursa of Fabricius. Although BCR expression is sufficient for bursal colonization, the role of BCR ligation in the later stages of bursal B cell lymphopoiesis remains elusive. To address this directly, we introduced a surface Ig-related construct with defined Ag specificity containing the Ag-binding portion of a lamprey variable lymphocyte receptor specific for PE fused to a truncated chicken μ-chain (VLRPETm) into developing chick embryos. VLRPETm expression supports bursal follicle colonization, clonal expansion, and Ig V gene diversification. VLRPETm-expressing B cells migrate to the periphery in the absence of the Ag starting from day 18 of embryogenesis. VLRPETm-expressing B cells declined rapidly in the bursa and periphery in the absence of Ag after hatch; however, intrabursal injection of PE prolonged survival of VLRPETm+ bursal and peripheral B cells. Intrabursal introduction of Ag increased migration of short-lived LT2+ B cells. Peripheral VLRPETm+ B cells were maintained following intrabursal PE application and contained both short-lived LT2+ and long-lived LT2− B cells. In the chicken bursa, the later stages of B cell development occur in the presence of gut-derived Ag; therefore, we conclude that Ag-mediated ligation of BCR in bursal B cells acts to positively select bursal B cells into both short-lived and long-lived peripheral B cell populations. The Journal of Immunology, 2014, 192: 3218–3227.

B lymphopoiesis in humans and rodents takes place in the fetal liver and, subsequently, in the bone marrow after birth. However, in many mammals and birds B cell development occurs in GALT, such as the appendix, Peyer’s patches, and the bursa of Fabricius (1–7). Although GALT B cell lymphopoiesis has been thought to be the dominant pathway in many nonrodent and primate mammalian species (3–5, 7), this has become less clear with a lack of demonstrable B cell development in porcine ileal Peyer’s patches (8, 9). Moreover, the demonstration of B cell development in the gut of postweaning mice suggests that bone marrow and GALT pathways of B cell development may coexist (10). B cell development in both bone marrow and GALT generates a diverse pool of B cells; however, there are molecular and anatomical differences between these models of B cell lymphopoiesis. Foreign Ag is excluded from the bone marrow environment, whereas B cell lymphopoiesis in GALT occurs in an environment that is exposed to foreign Ag after birth. This begs the question as to whether exogenous Ags play a role in the selection of B cells during B lymphopoiesis.

It is becoming apparent that avian B cell development can be divided into discrete stages. The early stages of chicken B cell development include colonization of bursal mesenchyme by B cell precursors, expression of a functional surface Ig (sIg) molecule, and colonization of bursal follicles, with subsequent cell proliferation and Ig diversification. Colonization of the bursal mesenchyme with a single wave of B cell precursors takes place during embryonic life in a germ-free environment and is independent of sIg expression (11). B cell precursors that have undergone successful Ig gene rearrangement, leading to functional BCR expression, migrate across the basement membrane and expand to form the bursal buds from which follicles develop (12, 13). Strong evidence supports a model in which BCR expression in the absence of ligation is necessary and sufficient to support transition through this developmental checkpoint (14–16).

The later stages of B cell development include redistribution to form cortical and medullary B cell compartments within the bursal follicle, the establishment of bursal subpopulations distinguished by differences in functional life span, and B cell migration to the periphery. These later stages coincide with the transportation of Ag through the bursal duct and into bursal follicles mediated by M cell–like follicle-associated epithelium (FAE) (17). Ectopic bursal grafts and bursae in which the bursal duct is ligated are colonized with B cell precursors during embryonic life but fail to continue development or diversification posthatch (18, 19). Although these results suggest the possibility that the presence of Ag in the medulla of bursal follicles can influence bursal B cell lymphopoiesis, there is no direct evidence supporting such a model.

We demonstrated previously that B cell precursors expressing a retrovirally transduced truncated sIgM (Tμ) that lacks Ag-binding capacity supports the early stages of B cell development. Similarly, a retrovirally transduced receptor in which the cytoplasmic domain of the chicken lgs is fused to the extracellular and transmembrane domains of mouse CD8α also provides equivalent support for early B cell development (20, 21). However, neither the Tμ nor CD8α: lgs construct supports the later stages of B cell development, with cells expressing either construct being rapidly eliminated within the first 10 d after hatch. Thus, BCR ligation by exposure to foreign
ligands, as distinct from BCR expression, might be required for the later stages of B cell development (22).

To address this question, we generated chimeric receptors in which the sequence of the diversity region of a lamprey variable lymphocyte receptor (VLR) is fused to the chicken Tm. This VLR, with specificity for PE (VLRPE), is expressed on the cell surface, and ligation of this receptor, when expressed in the chicken B cell lymphoma DT40, induces calcium mobilization. Retroviral transduction of this construct into developing B cell precursors supports the early stages of B cell development as effectively as does Tm, and, in the absence of cognate Ag, VLRPE-expressing B cells disappear rapidly after hatch. However, following intrabursal application of Ag, Ag-specific B cells are maintained in the bursa, their emigration to the periphery is enhanced, and Ag-specific B cells are maintained for extended periods of time in the periphery. To our knowledge, this provides the first direct demonstration of Ag-mediated positive selection of specific B cells during their development in a gut-associated lymphoid organ.

Materials and Methods

Generation of replication competent avian leukosis virus with splice acceptor (Bryan polymerase) subgroup B–VLRPE–Tm chimera

To generate a BCR with defined specificity, the diversity sequence of VLRPE (23) was fused to the chicken Tm sequence. The VLR diversity sequence was PCR amplified using Platinum PfX polymerase (Invitrogen, San Diego, CA) with the primer combination VLR3: 5′-ATTATTTGG-CCACCGCGATGTCCTCGACGT-3′ and VLR3′: 5′-ATTAATGCAGCG-GCCATTTGACGGGCTAGTG-3′ from the VLR-containing vector and cloned into Zero Blunt (Invitrogen). The cloned VLR was digested from this shuttle vector using EcoR1 and Eagl restriction sites embedded in the primers, and the VLR sequence ~600 bp was gel purified and cloned into the Eagl site of replication competent avian leukosis virus with splice acceptor (Bryan polymerase) subgroup B–VLRPET between the chicken H chain leader sequence and Tm (24).

RCAS(BP)B–VLRPET virus production and gene transfer in vivo and in vitro

The RCAS retroviral gene-transfer system was used to introduce VLRPET into chicken embryo fibroblasts and chicken embryos, as described (15). Line 0 chicken embryo fibroblasts (CEF) (Regional Poultry Research Laboratories, East Lansing, MI), cultured in IMDM (Life Technologies), (15). Line 0 chicken embryo fibroblasts (CEF) (Regional Poultry Research Laboratories, East Lansing, MI), cultured in IMDM (Life Technologies), were transfected with RCAS(BP)B–VLRPET plasmid by calcium chloride precipitation, as described (15). Within 7 d of transfection, essentially all CEFs expressed the retroviral containing chimeric receptor.

DT40 infection was performed as described previously (25). L chain–deficient DT40 chicken bursal B lymphoma cells were infected with RCAS(BP)B–VLRPET virus by coculturing serial dilutions of DT40 (starting at 5 × 10^6 cells) with semiconfluent transfected CEFs in 6 wells of a 12-well plate for 48 h in IMDM with 2% chicken serum. Nonadherent DT40 cells were harvested, expanded, and FACs sorted on a FACSaria (BD Biosciences, Mississauga, ON, Canada).

To generate chickens transduced with RCAS(BP)B–VLRPET, day-3 incubated B line chick embryos (ISA North America A Hendrix Genetics, Cambridge, ON, Canada) were inoculated with 1 × 10^6 CEFS (>98% VLRPET expressing) in 100 μl IMDM with 1% normal chicken serum.

Abs and flow cytometry

Surface expression of VLRPET was detected using R-PE (Columbia Biosciences, Columbia, MD) and/or anti-chicken μ (HY18) Ab. Ex vivo B cells were stained with the anti-chicken pan B cell marker anti-ChB6, anti-chicken μ (HY18), biotinylated or unlabeled anti-chicken Ig L chain, and R-PE (Columbia Biosciences), followed by FITC-conjugated anti-mouse Ig isotype Ab (Southern Biotechnology Associates, Birmingham, AL) or Streptavidin–PerCP (BD Biosciences), as described (14, 15). Samples were analyzed on a FACS caliber or sorted on a FACSaria (BD Biosciences).

For analysis of cellular DNA content, stained viable cells were sorted using a FACSaria, pelleted, and resuspended in Vindelov’s solution, as described (15). DNA content of the resulting nuclei was analyzed on a FACS caliber.

Calcium mobilization

Changes in cytosolic calcium concentrations following receptor ligation were detected in INDO-1 (Molecular Probes, Eugene, OR)-loaded cells, as described (26). Briefly, cells were washed with IMDM growth media and loaded with 10 μM final concentration of INDO-1 at 37 °C for 45 min in the dark, and 1 × 10^6 loaded cells were transferred to tubes. Samples were assayed on a BD LSR II and analyzed by BD FACSDiva software (BD Biosciences). Calcium mobilization was assessed following exposure of cells to anti-chicken H chain (HY18) or PE (26).

Ag application

Ag, typically 100 μl at 1 mg/ml in PBS, was applied to the anal lips of neonatal chicks and repeated the following day. Uptake of Ag was visualized by inclusion of 10 μl india ink into 1 ml Ag solution, and dissected bursae were photographed after 10 min or 1 h.

PCR amplification, gene-conversion analysis, and cloning of V gene sequences

Bursal and splenic cells were stained, and aliquots of 1500 cells of defined phenotype were sorted directly into PCR tubes. Cells were incubated in 15 μl 10 μg/ml proteinase K for 1 h at 50°C, followed by heat inactivation at 85°C for 20 min. VJ rearrangements were amplified using the primer combinations described previously (14, 15). All amplification reactions also contained the RAG5′ and RAG3′ combination of primers to amplify the single copy of chicken RAG2 sequence from genomic DNA as an internal standard. PCR products were electrophoresed, and bands were quantitated by scanning densitometry (Bio-Rad).

Gene-conversion events were assessed by restriction site analysis, as described, using the KpnI restriction enzyme (New England Biolabs) (27). VJ sequences were cloned into PCR2.1 (Invitrogen) and sequenced (TCAG, Toronto, ON, Canada).

Results

Generation of RCAS(BP)B–VLRPET–Tm–transduced chicken B cells

To generate Ig-related BCRs of defined specificity, we made use of Ag-specific lamprey VLRPE isolated from a yeast surface display library (23). The diversity region of the lamprey VLR was fused to the chicken Tm gene, using the chicken VH leader sequence. The resulting constructs were cloned into the RCAS(BP)B productive retroviral vector (Fig. 1A) and transfected into CEFs.

As we observed in other studies, surface expression of chicken Tm does not require coexpression of Igα and Igβ on CEFs and, under these circumstances, does not signal when cross-linked (25); RCAS(BP)B–Tm–transfected CEFs expressed high levels of surface Tm. CEFs transfected with RCAS(BP)B–VLRPET–Tm also expressed this construct at similar levels on the CEF surface (Fig. 1B). Staining with PE demonstrated that the RCAS(BP)B–VLRPET–Tm construct maintained the specificity of the original selected VLR (Fig. 1B).

VLRPET–Tm was expressed in the chicken B cell lymphoma DT40 by coculture of transfected CEFs with DT40 cells (Fig. 1B). VLRPET–expressing DT40 cells were purified to homogeneity by flow cytometric cell sorting and assessed for their ability to mobilize calcium in response to VLRPET ligation with soluble PE. As shown in Fig. 1C, cross-linking VLRPET on VLRPET–expressing DT40 cells resulted in levels of calcium mobilization equivalent to those seen following receptor cross-linking with anti-IgM Abs (Fig. 1C). Thus, when expressed on the B cell surface, VLRPET–transfected cells can be expressed on the cell surface in the absence of Igα/Igβ and VLRPET on VLRPET–transfected cells will associate with the Igα/Igβ complex if available.

Expression of VLRPET–Tm in the absence of ligation is sufficient to support the early stages of B cell development

Day-3 chicken embryos were inoculated with RCAS(BP)B–VLRPET–Tm–transduced CEFs and analyzed neonatally for the presence of
peripheral and bursal B cells expressing VLR\textsuperscript{PET} \textsubscript{\(\mu\)} and VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells lacking endogenous IgM were detected in the bursa, as well as peripherally in the spleen and PBLs, of \textsuperscript{30} chicks analyzed in three independent experiments (Fig. 2A). Cell cycle analysis showed that both sorted ex vivo bursal VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells, as well as endogenous sIg-expressing (IgL +/- VLR\textsuperscript{PET} \textsubscript{m}) B cells, were proliferating at a high rate, which is characteristic of neonatal bursal B cells (Fig. 2B). Thus, surface expression of a BCR, either sIg or VLR\textsuperscript{PET} \textsubscript{\(\mu\)}, is sufficient and required for the early stages of bursal cell development.

In addition, VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells showed a reduction in endogenous \(\text{L}^\text{chain} \) V to J rearrangement compared with B cells expressing endogenous \(\text{L}^\text{chain} \) M (data not shown). Semi-quantitative PCR of genomic DNA demonstrated that, in the absence of receptor ligation, expression of the VLR\textsuperscript{PET} \textsubscript{\(\mu\)} is sufficient to partially inhibit rearrangement of endogenous \(\text{L}^\text{chain} \) genes in developing B cells. The levels of inhibition observed were similar to those seen in developing B cells expressing either the \(\text{T}^\text{\(\mu\)} \) receptor or the CD8\(\alpha\):Ig\(\alpha\) receptor (14, 15).

To assess gene conversion at the Ig L chain locus, we performed restriction digests and sequence analysis of VJ\(\text{L} \) sequences from sorted VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells from the bursa and spleen of RCAS-VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-transduced chicks. The germline V\(\text{L} \) sequence contains a KpnI restriction site that is partially lost upon gene conversion during the diversification of the rearranged \(\text{L}^\text{chain} \) allele (15, 27). Therefore, KpnI digests were performed on VJ\(\text{L} \) sequences amplified from sorted VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells (Fig 2C). To ensure that digests were performed to completion, the pUC18 plasmid containing a KpnI site was included in all reactions, as indicated. VJ\(\text{L} \) sequences from bursal VLR\textsuperscript{PET} \textsubscript{\(\mu\)} B cells were largely resistant to digestion with KpnI as were VJ\(\text{L} \) sequences from bursal cells expressing endogenous sIg. Therefore, expression of the VLR\textsuperscript{PET} \textsubscript{\(\mu\)} supports the induction of gene conversion as efficiently as does \(\text{T}^\text{\(\mu\)} \) or CD8\(\alpha\):Ig\(\alpha\) (14, 15). At days 18–19, most splenic B cells are prebursal, and the bursal microenvironment is required for the onset of gene conversion. Consistent with this, VJ\(\text{L} \) sequences derived from splenic VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells were heavily digested with KpnI. This suggests that splenic VLR\textsuperscript{PET} \textsubscript{\(\mu\)}-expressing B cells have undergone less gene conversion than age-matched bursal B cells. This observation was confirmed by sequencing cloned VJ\(\text{L} \) genes from the relevant B cell populations (data not shown).

Intrabursal exposure to Ag increases B cell migration to the periphery

B cell migration from bursa to the periphery starts at about day 18 of embryonic life in chickens and continues posthatch until sexual maturity. Within the bursa after hatch, B cells redistribute and generate cortical and medullary populations separated by a cortico-medullary junction that develops from the basement membrane. This redistribution coincides with transportation of the contents of the gut to the bursal medulla by FAE. Whether transport of Ag has any direct impact on the redistribution and migration of B cells to the periphery remains unclear.
The contents of the cloaca are transported to the bursal duct by reverse peristalsis. Therefore, we applied Ag into the cloaca to mimic the physiological route of gut exposure to Ag. To visualize and confirm the effective transport of the injected material to the bursa lumen, the PE Ag was mixed with india ink. After 10 min, carbon particles were not detected in the cells of the FAE. However, 1 h after application, carbon particles were detected in the follicular-associated epithelial cells that map to the organization of B cell follicles (Fig. 3).

To assess the consequences of BCR ligation on the fate of chicken bursal B cells, we introduced PE intrabursally into chicks that had been transduced with RCAS(BP)B-VLRPETm. The penetration of the transduced VLRPETm was assessed on PBLs from day-1 neonates. Chicks with similar VLRPETm-expressing B cell populations were divided into two groups, and 100 μl of PE/india ink solution was applied daily onto the anal lips of VLRPETm-transduced neonates for 2 d. Two days after PE application, the frequency of peripheral blood VLRPETm-expressing B cells was assessed. Although there was a slight trend toward increased levels of VLRPETm-expressing B cells following PE exposure, the increase was not significant (Fig. 3B). At a minimum, this result clearly indicates that intrabursal application of Ag does not mediate negative selection among peripheral B cells.

It was shown that >90% of bursal emigrants to the periphery of a 3-wk-old chickens are short-lived B cells, with a life span of 1–2 d, which can be identified by the expression of a marker defined by the LT2 Ab (28). Subsequent to hatch, LT2 expression is largely maintained among bursal B cells, although a clear population of LT2lo/2 cells emerges. Among peripheral B cells, the frequency of LT2+ emigrants increases with time, such that by 1 mo of age there are clearly defined populations of peripheral LT2+ and LT2− cells. At this stage, although the LT2− peripheral cells have been shown to be short-lived bursal emigrants, the great majority of LT2− peripheral cells are long-lived bursal emigrants.

Although intrabursal application of PE Ag did not substantially affect the overall frequency of peripheral VLRPETm-expressing PBL B cells, we used the LT2 Ag to specifically assess whether the intrabursal application of Ag affected the rate of emigration of short-lived B cells from the bursa (Fig. 4A). In chicks exposed to intrabursal PE, we observed a higher frequency of LT2+ VLRPETm-expressing B cells in the PBLs compared with control chicks. In contrast, in the same chicks, there was no difference in the frequency of LT2+ B cells expressing endogenous slg in the PBLs of the Ag-treated versus control chicks (Fig. 4A, 4B). Thus, increased levels of VLRPETm+ LT2− peripheral B cells were specific for the Ag treatment. This is suggestive of Ag-driven emigration of B cells from the bursa.

In the presence of intrabursally administered PE, the frequency of LT2− PBL B cells was approximately equivalent when com-
FIGURE 3. Intrabursal Ag treatment does not induce negative selection of peripheral B cells. (A) PE/india ink mixture was introduced into the bursal lumen. Uptake of the colloidal india ink by FAE was visualized as black staining on the follicular epithelium 10 min (Aiv) or 1 h (Ai, Aii, and Aiii) after application. Original magnifications are: ×3 (Ai), ×9 (Aii), ×30 (Aiii), ×25 (Aiv). (B) Chick embryos were inoculated with VLR<sup>PE</sup>T<sub>µ</sub>- or T<sub>µ</sub>-transduced CEFs, and PE/india ink was introduced into the bursal lumen of day-1–2 VLR<sup>PE</sup>T<sub>µ</sub>-transduced chicks. PBLs of day-3–5 chicks were assessed by flow cytometry for the frequency of VLR<sup>PE</sup>T<sub>µ</sub>− or T<sub>µ</sub>-expressing ChB<sup>+</sup> B cells. Horizontal bars represent the mean of each group.

Ag-dependent maintenance of B cells

In the bursa, cells expressing either T<sub>µ</sub> or CD8α:1gα were maintained for only ∼10 d in RCAS-T<sub>µ</sub>− or RCAS-CD8α:1gα-transduced chicks, respectively (20, 21). Similarly, in the absence of Ag, VLR<sup>PE</sup>T<sub>µ</sub>-expressing B cells in the bursa disappeared over the first few weeks of life, such that by day 21, <1% of VLR<sup>PE</sup>T<sub>µ</sub>-expressing bursal cells remained (Fig. 5A). Thus, in the absence of cognate Ag, the VLR<sup>PE</sup>T<sub>µ</sub> construct behaves equivalently to those provided within the normal bursal microenvironment to B cells expressing endogenous slg.

Ag-dependent maintenance of B cells in the absence of intrabursal PE

In the absence of intrabursal PE, the proportion of LT<sub>2</sub>+ VLR<sup>PE</sup>T<sub>µ</sub>− B cells was significantly lower than the proportion of LT<sub>2</sub>+ slg<sup>+</sup> B cells (Fig. 4C, right panel). This suggests that signals provided to VLR<sup>PE</sup>T<sub>µ</sub>− bursal cells in the presence of PE are functionally equivalent to those provided within the normal bursal microenvironment to B cells expressing endogenous slg.

Among the peripheral B cells in the absence of intrabursal Ag, few, if any, VLR<sup>PE</sup>T<sub>µ</sub>-expressing cells were observed at day 21 (Fig. 6A). Peripheral B cells at this stage include both LT<sub>2</sub>− and LT<sub>2</sub>+ populations, with the LT<sub>2</sub>− population disappearing following bursectomy (Fig. 6A). In contrast, following Ag application in the bursa, VLR<sup>PE</sup>T<sub>µ</sub>-expressing B cells were maintained in the PBLs for extended periods of time (Fig. 6B, top row). These VLR<sup>PE</sup>T<sub>µ</sub>-expressing B cells also contained both LT<sub>2</sub>− and LT<sub>2</sub>+, with the LT<sub>2</sub>− VLR<sup>PE</sup>T<sub>µ</sub>-expressing population disappearing rapidly within 7 d postbursectomy (Fig. 6A, middle row). Therefore, at least some peripheral VLR<sup>PE</sup>T<sub>µ</sub>− B cells are recent bursal emigrants whose emigration from the bursa is dependent upon the maintenance of VLR<sup>PE</sup>T<sub>µ</sub>− bursal B cells, which, in turn, is driven by the presence of intrabursal Ag.

Not all VLR<sup>PE</sup>T<sub>µ</sub>− peripheral B cells expressed LT<sub>2</sub> (Fig. 6), and some VLR<sup>PE</sup>T<sub>µ</sub>− PBL B cells in intrabursal PE-treated VLR<sup>PE</sup>T<sub>µ</sub>-transduced chicks clearly fall into the long-lived emigrant population, being maintained for ≥21 d following bursectomy (Fig. 6B, bottom row). This was confirmed by assessing the frequency of VLR<sup>PE</sup>T<sub>µ</sub>− splenic B cells 28 d after surgical bursectomy in VLR<sup>PE</sup>T<sub>µ</sub>-transduced chickens that had been treated with PE intrabursally (Fig. 7). Despite the complete disappearance of splenic LT<sub>2</sub>− B cells, a significant population of PE-binding B cells was maintained. Thus, some VLR<sup>PE</sup>T<sub>µ</sub>− peripheral B cells are long-lived LT<sub>2</sub>− emigrant B cells. Therefore, we conclude that the presence of specific Ag in the bursa has the capacity to ligate Ag-specific receptors on VLR<sup>PE</sup>T<sub>µ</sub>-expressing B cells, resulting in positive selection of B cells. Therefore, at least some peripheral VLR<sup>PE</sup>T<sub>µ</sub>− B cells are recent emigrants from the bursa or whether they are longer-lived cells whose presence is maintained in some way by exposure to Ag in the bursa.
maintained viability within the bursa and sustained emigration to the periphery (Fig. 8).

Discussion

Although B cell development in some mammalian species, such as human and mouse, occurs in an environment free of exogenous Ag, B cell development in other species occurs in GALT. This provokes the question as to whether the presence of Ag might impact B cell lymphopoiesis in the “so-called” GALT species (22).

The current study addressed the consequences of gut-derived Ag exposure on bursal B cell fate using the RCAS retroviral vector (29) to introduce Ag-specific chimeric BCR into developing chicken embryos. Because retroviral transduction is not 100%, this system allows for direct comparisons to be made between cells expressing the transduced BCR and cells expressing endogenous sIg (15, 24).

We demonstrated previously that BCR expression in the absence of ligation is sufficient to support B cell transition through the Ig-selection checkpoint, leading to productive bursal colonization (14–16, 24, 30–32). Similarly, we show in this study that the VLRPET\textsubscript{m} receptor supported all early stages of B cell development in the bursa, characteristic of B cells passing the Ig-selection checkpoint. Thus, in the absence of Ag, VLRPET\textsubscript{m}+ B cells colonize bursal follicles, undergo rapid proliferation, and undergo Ig diversification by gene conversion.

However, BCR expression in the absence of ligation is not sufficient to support the long-term maintenance of bursal B cells (20). In fact, in the absence of PE, VLR\textsuperscript{PET}\textsubscript{m}+ B cells disappear from the bursa within the first 10 d after hatch (Fig. 5). Disappearance of B cells expressing the T\textsubscript{m} chimeric receptor was shown to be independent of competition with B cells expressing endogenous BCR (20). Moreover, in the chicken, all Ig gene rearrangement occurs during embryonic life, and the entire B cell repertoire is derived from cells expressing sIg by embryonic day 17 (11, 33). Therefore, the disappearance of cells expressing VLR\textsuperscript{PET}\textsubscript{m} cannot simply be due to their replacement by newly developing B cells undergoing Ig gene rearrangement.

Ab-mediated cross-linking of chimeric CD8\textsubscript{a}:Ig\textsubscript{a} BCR receptors in the bursa prolonged the survival of CD8\textsubscript{a}:Igs-expressing B cells (21), although the physiological relevance of Ab-mediated cross-linking of BCR can be questioned. However, the VLR\textsuperscript{PET}\textsubscript{m} construct allowed us to assess the role of Ag and specific BCR ligation in chicken B cell development. Typically, BCR cross-linking on immature B cells has been considered to induce tolerance (34, 35). Indeed, we provided evidence that exposure of developing B cells in the chick embryo to a BCR ligand...
introduced i.v. induced a BCR-signaling–dependent deletion of B cells (36). Conversely, we show in this study that intrabursally administered Ag maintains bursal B cells. Thus, we conclude that the route of the Ag administration changes the outcome of BCR ligation. This could be due to the context in which the Ag is exposed to costimulatory receptors expressed by the FDCs themselves. In contrast, developing prebursal B cells encountering i.v.-injected PE may receive signals through BCR in the absence of costimulatory signals provided by appropriate Ag presentation. Thus, gut-derived Ag, such as the PE administered in the current experiments, could induce positive selection of PE-specific B cells, whereas i.v.-injected Ag results in B cell deletion. It would be intriguing to examine the effect of specific Ag on chicken B cell development in embryos derived from IgG-deficient hens (37), in which intrabursally applied Ag would not be presented in the context of immune complexes.

Normal slg+ bursal B cells are maintained in the bursa until the involution of the organ at ~6 mo of age. Bursal duct ligation (19, 38) and ectopic grafting of the bursa to sites in which there is no direct contact with the gut (18) result in the failure of continued bursal B cell development after hatch, suggesting that the signals supporting slg+ bursal cells are gut derived. We show in this study that maintenance of bursal B cells is a consequence of BCR ligation, suggesting that such maintenance signals are not simply gut-derived mitogens acting through non-IgRs.

The nature of the ligand for the BCR of normal slg+ bursal cells remains unclear. Gut-derived bacterial superantigen positively selected rabbit B cells of a defined BCR allotype, independent of receptor specificity (7, 39). Nonetheless, this superantigen clearly signals through slg. In our experiments, it is highly unlikely that a gut-derived superantigen would bind the VLR of the VLRPETm construct given that the lamprey VLR is not a member of the Ig supergene family. However, although we showed that Ag-mediated BCR ligation leads to the support of bursal B cells, we cannot distinguish between the presence of a B cell superantigen activating B cells through BCR, independent of their specificity, or the presence of a multiplicity of gut-derived Ags, each acting on Ag-specific B cells; indeed, these models are not mutually exclusive.

After hatch, some bursal cells migrate back across the basement membrane to form the follicular cortex, whereas Ag taken up through the FAE is retained in the follicular medulla (17, 32). Bursal cells expressing either Tm or the chimeric CD8α:Igs BCR fail to migrate into the cortex (25), although there is some evidence that CD8α:Igs+ bursal cells migrate into the cortex when exposed to anti-CD8α Abs intrabursally (21). It is tempting to suggest that, following intrabursal exposure to PE, VLR PETm+-bursal cells are maintained and colonize the follicular cortex, although evidence supporting the latter is lacking. Migration of VLR PETm+-expressing B cells to the follicular cortex would place these cells into a physiologically distinct compartment within the bursa from which gut-derived Ags are excluded (32). Thus, although Ag-induced proliferation may be initiated in the follicular medulla, the transit of these cells into the Ag-free cortical environment may limit the proliferative burst.

The bursal follicular cortex has been identified as a site of Ig repertoire diversification by gene conversion, based on the high levels of activation-induced cytidine deaminase (AID) seen in cortical bursal cells (40). In the case of cells expressing VLR PETm, diversification of the VLR PETm receptor by gene conversion could not occur because specificity is determined by the retrovirally transduced construct. However, among normal bursal cells, the high levels of cortical AID expression suggest that exposure to Ag in the follicular medulla might induce migration into the cortex, followed by continued proliferation and repertoire diversification by gene conversion. This would generate a situation that, at first sight appears paradoxical but would represent an Ag-driven generation of a naive B cell...
repertoire and preclude the possibility of the naive B cell repertoire becoming restricted to those specificities recognizing gut-derived Ags. This possibility is supported by the observation that SRBCs introduced into the normal neonatal chick bursa through the bursal lumen do not result in a detectable increase in SRBC-binding bursal B cells (41). Indeed, not only does intrabursal Ag fail to result in increased numbers of Ag-binding bursal B cells, it also fails to induce Ag-specific Ab responses (42).

Bursal B cells migrate to the periphery where they form the Ag-responsive mature B cell population. This migration is initiated during the later stages of embryonic life, and we show in this study that this can occur in the embryo independent of BCR ligation, because VLR<sup>PET</sup><sub>m</sub>-expressing B cells migrate to the periphery in the absence of intrabursal PE. Nonetheless, intrabursal BCR ligation clearly enhances the emigration of bursal cells to the periphery.

We showed previously that bursal emigrants fall into two phenotypically and functionally distinct populations. Those B cells expressing the LT2 marker are short-lived bursal emigrants and are distinct from a longer-lived population of LT2<sup>−</sup>bursal emigrants (28, 43). In the presence of PE, the proportion of LT2<sup>+</sup> VLR<sup>PET</sup><sub>m</sub>+ cells that is found in the periphery is directly proportional to the frequency of LT2<sup>+</sup> sIg<sup>+</sup> cells in the periphery (Fig. 4C). In contrast, in the absence of PE, the proportion of LT2<sup>+</sup> VLR<sup>PET</sup><sub>m</sub>+ cells in the periphery is substantially lower than the proportion of LT2<sup>−</sup> sIg<sup>+</sup> peripheral B cells. Therefore, we can conclude that the signals required to support the emigration of short-lived LT2<sup>+</sup> sIg<sup>+</sup> cells from the bursa are functionally equiva-

**FIGURE 6.** Intrabursal Ag maintains peripheral LT2<sup>+</sup> short-lived and LT2<sup>−</sup> long-lived postbursal cells. Chick embryos were inoculated with VLR<sup>PET</sup><sub>m</sub>-transduced CEFs, and PE/india ink was applied to the bursal lumen of day-1–2 chicks, followed by surgical bursectomy on day 21 posthatch. VLR<sup>PET</sup><sub>m</sub>-PBL B cells in control (A) or PE-treated (B) chicks were assessed before and after bursectomy. VLR<sup>PET</sub>m<sup>+</sup> B cells were assessed for LT2 expression by staining PBLs for μ, PE, LT2, and ChB6. Data are representative of 50,000 cells gated on forward scatter and side scatter, and PE-binding graphs are gated on ChB6<sup>+</sup> B cells. LT2 plots were gated on ChB6<sup>+</sup> PE<sup>+</sup> or ChB6<sup>+</sup> PE<sup>−</sup> cells, representative of three chicks analyzed.
emigration from the bursa. Support of the later stages of chicken B cell development, including cells. Again, this is fully consistent with a role for BCR ligation in Ag-binding graphs are gated on ChB6+ B cells, and LT2 graphs are gated forward scatter, side scatter, and live cells and represent 50,000 events. PE on ChB6+, ChB6+ PE+, or ChB6+ PE− B cells, as indicated.

Intrabursal exposure to PE induces higher levels of LT2− VLRPET− B cells, as indicated. Our finding that intrabursal PE induces higher levels of LT2− VLRPET− bursal cells is consistent with a role for BCR ligation in support of the later stages of chicken B cell development, including emigration from the bursa.

In summary, the peripheral B cell compartment would contain a diverse population of naive LT2− B cells that have matured through the follicular cortex and an additional longer-lived population of LT2− cells enriched for specificities present in the environment. Under these circumstances, BCR ligation in the medulla of bursal follicles could lead to both the generation of the diversified pool of naive B cells following redistribution to follicular cortex and subsequent emigration, as well as the generation of a longer-lived Ag-enriched peripheral population following emigration directly from the follicular medulla.

Intrabursal exposure to PE additionally generates a population of LT2− VLRPET− peripheral B cells that survives for extended periods following surgical bursectomy (Figs. 6, 7). Thus, BCR ligation may induce a transition from short-lived to long-lived B cells. Although short-lived LT2− bursal emigrants are derived predominantly from the follicular cortex, longer-lived LT2− bursal emigrants are likely derived from the follicular medulla (43).

We previously argued that because the follicular medulla is a site of gut-derived Ag, long-lived LT2− bursal emigrants may represent a population of B cells that have experienced Ag exposure. Therefore, the peripheral B cell compartment would contain a diverse population of naïve B cells and an additional longer-lived population of LT2− cells enriched for specificities present in the environment. Under these circumstances, BCR ligation in the medulla of bursal follicles could lead to both the generation of the diversified pool of naïve B cells following redistribution to follicular cortex and subsequent emigration, as well as the generation of a longer-lived Ag-enriched peripheral population following emigration directly from the follicular medulla.

In summary, the peripheral B cell compartment would contain a diverse population of naïve LT2− B cells that have matured through the follicular cortex and an additional longer-lived population of medullary-derived LT2− cells enriched for specificities present in the environment (Fig. 8). Under these circumstances, BCR ligation in the medulla of bursal follicles could lead to both the generation of the diversified pool of naïve B cells following their redistribution to the follicular cortex and subsequent emigration and the generation of a longer-lived Ag-enriched peripheral population following emigration directly from the follicular medulla.

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Disclosures

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References


