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Autoreactive B Cell Receptors Mimic Autonomous Pre-B Cell Receptor Signaling and Induce Proliferation of Early B Cells

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SUMMARY

The majority of early immature B cells express autoreactive B cell receptors (BCRs) that are, according to the current view, negatively selected to avoid the production of self-reactive antibodies. Here, we show that polyreactive BCRs, which recognize multiple self-antigens, induced autonomous signaling and selective expansion of B cell precursors in a manner comparable to the pre-BCR. We found that the pre-BCR was capable of recognizing multiple self-antigens and that a signaling-deficient pre-BCR lacking the non-Ig region of the surrogate-light-chain component λ5 was rescued by the complementarity-determining region 3 derived from heavy chains of polyreactive receptors. Importantly, bone marrow B cells from mice carrying Ig transgenes for an autoreactive BCR showed increased cell-cycle activity, which could not be detected in cells lacking the transgenic BCR. Together, the pre-BCR has evolved to ensure self-recognition because autoreactivity is required for positive selection of B cell precursors.

INTRODUCTION

B lymphocytes produce highly diverse antibodies that are essential for the body's defense against pathogens. Antibody diversity is achieved by random recombination of immunoglobulin (Ig) variable (V), diversity (D), and joining (J) gene segments in developing B cell precursors (Rolink et al., 1999; Schlissel, 2003). Although this process is essential to yield highly diverse antigen receptors, it can lead to the generation of B cells with self-reactive receptors that need to be removed from the repertoire by selection mechanisms (Silverstein, 2001). Experiments using transgenic mouse models suggested that negative-selection mechanisms such as clonal deletion, receptor editing, and anergy may counteract the production of self-reactive antibodies (Chen et al., 1997; Goodnow et al., 1988; Nemazee and Burki, 1989; Tiegs et al., 1993; Tze et al., 2000). Deletion (i.e., elimination of autoreactive cells) and receptor editing are considered to be the main mechanisms of establishing central tolerance in the bone marrow. However, the magnitude of each in accomplishing immunological tolerance is unclear (Nemazee and Weigert, 2000). Receptor editing involves secondary recombination of IgL genes, which results in alteration of antigen-receptor specificity and has been shown to be an efficient mechanism that enables autoreactive B cells to participate in B cell development as successfully as nonautoreactive counterparts (Casellas et al., 2001; Halverson et al., 2004; Oberdoerffer et al., 2003; Retter and Nemazee, 1998; Tze et al., 2005). These data suggest that effective receptor editing is the main mechanism for the removal of autoreactive B cell antigen receptors (BCRs) from the repertoire of immature B cells and that autoreactive cells are deleted only if editing is not possible.

Interestingly, the majority of BCRs of early immature B cells of healthy individuals display marked polyreactivity (Wardemann et al., 2003). This high proportion of polyreactive antibodies has been ascribed to random assembly of antibody-combining sites by V(D)J recombination, leading to a very diverse group of antigen receptors including some with self-reactivity. However, reports using transgenic mice expressing autoreactive receptors showed that the presence of self-antigens (self-Ags) induced positive selection of all B cell subsets (Hayakawa et al., 1999; Koenig-Marrony et al., 2001). According to the current view, the pre-BCR acts in a cell-autonomous manner whereby the surrogate light chain induces ligand-independent pre-BCR crosslinking and subsequent autonomous signaling that leads to expansion, which is required for positive selection of B cells expressing a productively recombined heavy chain. In contrast, autoantigen-induced crosslinking and subsequent BCR signaling on immature B cells is thought to induce negative selection (King and Monroe, 2000). This view raises the question as to how early immature B cells, which just started BCR expression, can discriminate between signals from auto-crosslinked pre-BCRs, which induce positive selection, and signals from antigen-crosslinked BCR, which result in negative selection.

Using an in vitro system for inducible activation of the adaptor protein SLP-65, a central signaling protein downstream of the BCR, we have recently proposed that polyreactive receptors confer an initial selective advantage, which results in the expansion of corresponding cells in the bone marrow (Meixlsperger et al., 2007). In this scheme, polyreactive BCRs act similarly to the pre-BCR, thereby inducing survival and expansion of early

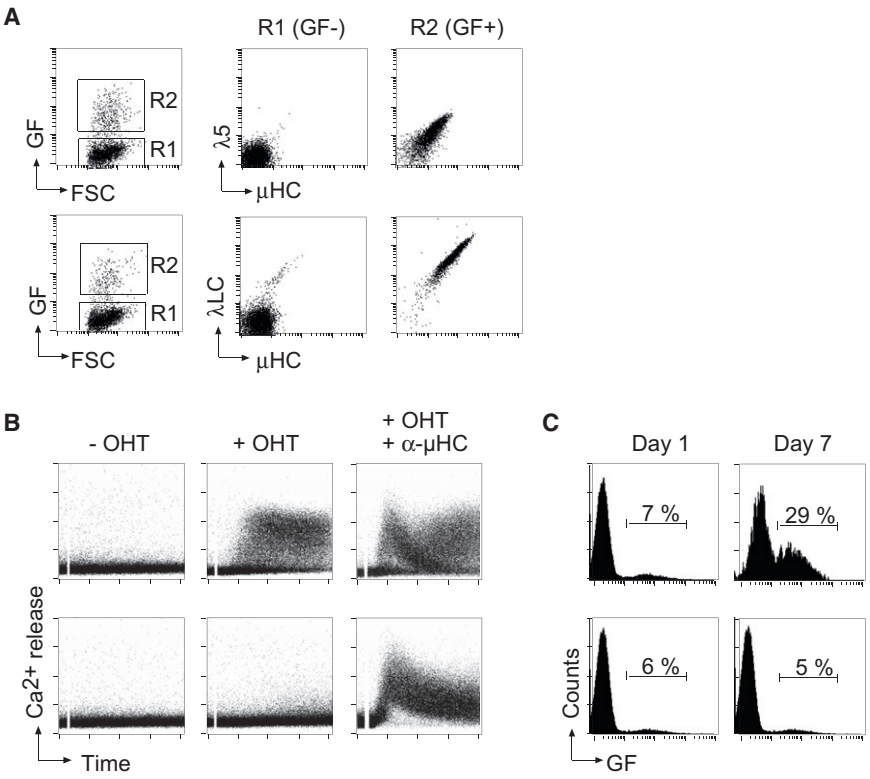

Figure 1. The BiFC System for Murine Pre-BCR and BCR Expression

(A) FACS analysis of pre-BCR expression (top) and BCR expression (bottom) on day 1 after transduction. SLP-65, λ5, RAG2 triple-deficient cells expressing ERT2-SLP-65 were transduced with the respective vector pairs depicted in Figure S3A. The first plot shows FSC versus GF with GF being a marker for HC and LC double-positive cells. Receptor surface expression is depicted for GF-negative (R1) and GF-positive cells (R2).

(B) Ca^{2+} measurement of the pre-BCR positive (top) and BCR positive cells (bottom) shown in (A). Cells were either mock stimulated with EtOH (−OHT), or ERT2-SLP-65 was activated by adding 1 μM OHT (+OHT). The right panels display stimulation with 1 μM OHT and 10 μg/ml α-μHC antibody.

(C) Percentages of GF-positive cells at day 1 and day 7 after transduction. Representative data of more than five independent experiments.

immature B cells and allowing secondary recombination of the IgL genes, which is also known as receptor editing.

Here, we test this hypothesis and show that the pre-BCR and polyreactive receptors are functionally equivalent and that they share structural elements involved in multispecific recognition of self-Ags. We present a concept for early B cell development suggesting that autoantigen-crosslinked BCRs recapitulate pre-BCR signaling to activate secondary IgL-gene recombinations and accomplish receptor editing.

RESULTS

Autonomous Signaling by the Pre-BCR

To compare the signaling capacity of autoreactive and nonautoreactive receptors, we used bone-marrow-derived cells from mice deficient for Rag2, λ5, and SLP-65 (triple-deficient pre-B cells [Figure S1 available online]) as a reconstitution system to introduce combinations of heavy and light chains (HCs and LCs, respectively) (Meixlsperger et al., 2007). To track those cells that coexpress heavy and light chains, we constructed two retroviral vectors containing an internal ribosome entry site (IRES) followed by sequences encoding a fusion between the leucine zipper of GCN4 (a yeast transcription factor) and either an N-terminal fragment of YFP or a C-terminal fragment of CFP (Figure S2). Previous reports showed that coexpression of such fusion proteins leads to bimolecular fluorescence complementation (BiFC) mediated by the leucine zippers (Hu and Kerppola, 2003). Indeed, single transduction did not lead to detectable fluorescence, whereas cotransduction of the two retroviruses led to green fluorescence (GF) detectable in the FACS FITC channel (Figure S2). We next tested the BiFC system with murine μHC (B1-8) together with either its corresponding λLC (B1-8) or with murine λ5 cloned into the respective BiFC vectors (Figure S3A). Staining for μHC and either λ or λ5 revealed that all cells expressing both proteins (i.e., BCRs or pre-BCRs) displayed green fluorescence (Figure 1A). As expected, cells transduced with plasmids encoding the mouse BCR expressed large amounts of the receptor on their surface as compared with those expressing the pre-BCR. This low pre-BCR expression is thought to be a consequence of constitutive pre-BCR signaling that leads to receptor downmodulation (Ohnishi and Melchers, 2003). In line with this constitutive pre-BCR signaling, our results show that activation of SLP-65 function with a 4-hydroxytamoxifen (OHT)-inducible form of SLP-65 is sufficient to induce robust calcium mobilization in cells expressing the pre-BCR, whereas cells expressing the BCR required receptor crosslinking (Figure 1B). Signaling via the pre-BCR is thought to induce selective enrichment of cells with a productively recombined μHC. Therefore, we tested whether reconstitution of pre-BCR expression in triple-deficient cells resulted in enrichment of the respective cells. We observed that indeed, pre-BCR expression confers a growth advantage because the proportion of pre-BCR expressing cells increased by more than 4-fold after 1 week of cultivation in vitro, whereas cells expressing BCR did not enrich under these conditions (Figure 1C). Taken together, our results showed that expression of the pre-BCR is sufficient to induce autonomous receptor signaling in our cells.

Autonomous Signaling by Polyreactive BCRs

To analyze polyreactive BCRs in our cellular system, we constructed chimeras of the murine μHC constant region with variable (VDJ) regions of polyreactive antibodies from nonselected, bone-marrow-derived B cells of healthy human donors (Figure S3B). We chose two polyreactive antibodies (BCR62 [eiBC62] and BCR115 [eiGO115]) and compared them to a nonautoreactive antibody derived from a selected, peripheral B cell

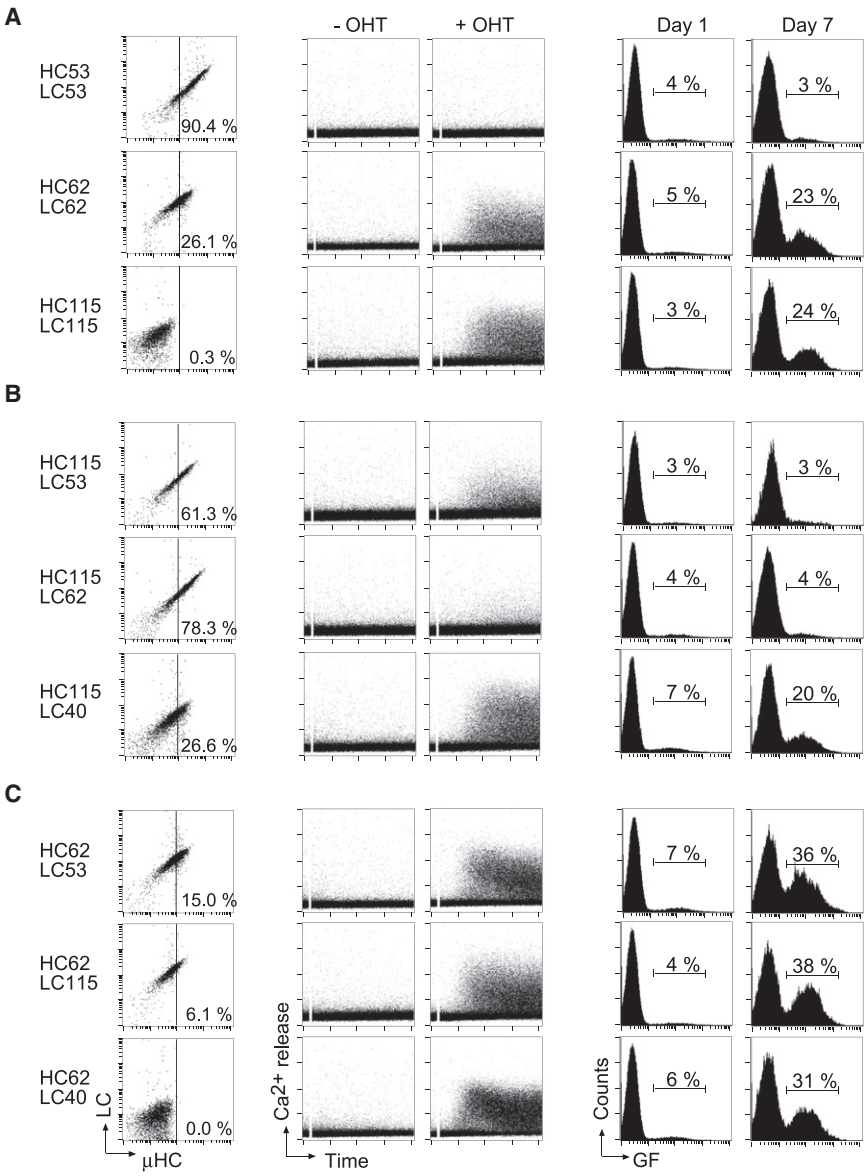

Figure 2. Cells Expressing Polyreactive BCRs Behave Like Cells Expressing Murine pre-BCR

(A) μHC versus LC FACS analysis (left). Triple-deficient cells expressing ERT2-SLP-65 were transduced with the respective vector pairs depicted in Figure S3B and analyzed on day 1 after transduction. Receptor expression is shown for the GF-positive cells. The numbers indicate the percentage of μHC high-expressing cells. Cells positive for ERT2-SLP-65 and respective receptors were subjected to Ca^{2+} measurement without (−OHT) or with the addition of 1 μM OHT (+OHT). Right panels shows percentages of GF-positive cells at day 1 and day 7 after transduction.

(B) FACS analysis, Ca^{2+} measurement, and percentages of GF-positive cells as in (A) for the indicated combinations of HC115 and various LCs.

(C) FACS analysis, Ca^{2+} measurement, and percentages of GF-positive cells as in (A) for the indicated combinations of HC62 and various LCs. Representative data of more than five independent experiments.

(BCR53 [mGO53]) (Wardemann et al., 2004). Introduction of BCR53 into triple-deficient pre-B cells resulted in a large amount of receptor expression on the cell surface, whereas introducing BCR62 or BCR115 led to reduced receptor expression (Figure 2A). This low receptor expression, particularly in the case of BCR115, was comparable to murine pre-BCRs, suggesting that polyreactive receptors may indeed act similarly to the pre-BCR.

To further compare the polyreactive and nonpolyreactive receptors with the pre-BCR, we investigated their ability to induce Ca^{2+} flux after induction of SLP-65 function. Similar to cells expressing murine BCR, cells expressing BCR53 displayed no Ca^{2+} flux upon SLP-65 induction, whereas those expressing the polyreactive BCRs showed efficient Ca^{2+} mobilization (Figure 2A). Treatment with μHC-specific antibodies led to efficient Ca^{2+} mobilization in cells expressing BCR53, suggesting that this receptor is functional and requires external crosslinking to activate signaling (Figure S4). These data suggest that polyreactive BCRs, despite being associated with a conventional LC, are capable of constitutive signaling and thus behave like the pre-BCR and clearly distinct from nonpolyreactive ones. Remarkably, analysis of the different chimeric BCRs showed that cells expressing BCR53 did not enrich, whereas those expressing the highly polyreactive species BCR62 and BCR115 showed enrichment comparable to that of cells expressing murine pre-BCR (Figure 2A). Importantly, expression of the polyreactive receptors in triple-deficient cells, which were stably reconstituted with SLP-65, led to similar results suggesting that the selection induced by the murine pre-BCR or polyreactive BCRs was not a specific result of SLP-65 deficiency (data not shown). These results suggest that polyreactive BCRs act like pre-BCRs and activate signaling, thereby conferring an initial growth advantage to respective B cells, and such a growth advantage leads to enrichment of early immature cells expressing a polyreactive BCR on their surface.

Light-Chain Exchange Inhibits Autonomous Signaling

One process of avoiding the generation of autoreactive antibodies is receptor editing. In this case, secondary recombination at the LC gene loci generates other LCs, which substitute the initial ones and can lead to elimination of the multireactivity encoded by the HC. One (eiBC62) of the highly polyreactive antibodies we chose for our experiments has been shown to be refractory to inhibition by other LCs, whereas the other one (eiGO115) was responsive (Wardemann et al., 2004). We wanted to know whether we could monitor elimination of the autoreative

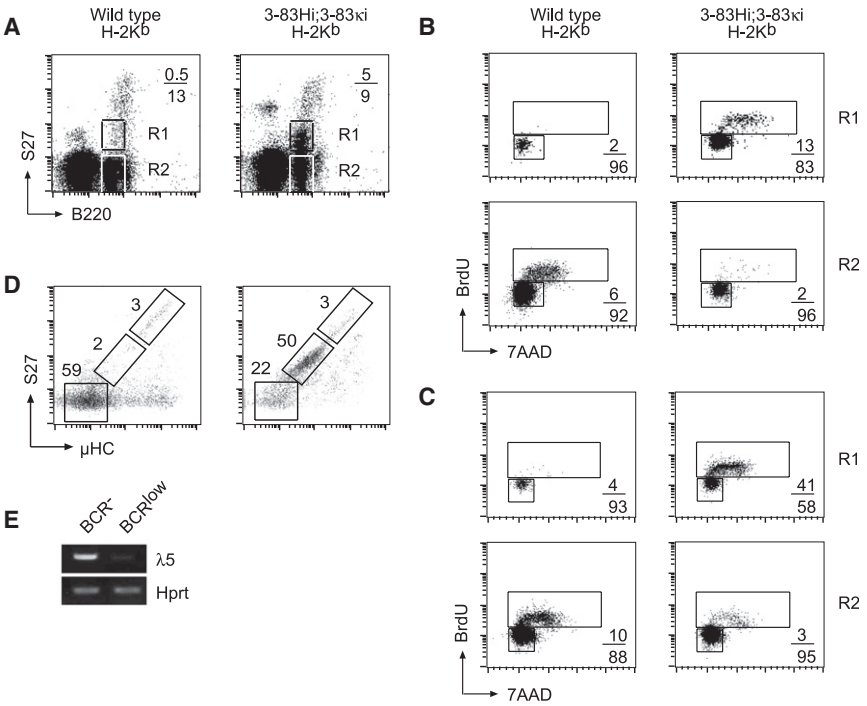

Figure 3. Active Cycling of B Cells Expressing an Autoreactive Transgenic Receptor

(A) Intracellular FACS analysis of bone-marrow cells from 3-83Igi (3-83Hi;3-83κi) and wild-type mice with B220 versus S27 (recognizes the LC of 3-83κi). The background of the mice is indicated. B cells showing low or negative S27 staining are marked as R1 and R2, respectively. The numbers indicate the percentages of the R1 and R2 cells in the bone marrow.

(B) BrdU versus 7AAD (DNA content) FACS profiles of in vivo-labeled cells. Mice were injected with BrdU 1 hr prior to the isolation of bone-marrow cells and subsequent staining with B220 and S27. BrdU and 7AAD are shown for R1 and R2 as indicated. Cycling cells (BrdU positive) and noncycling cells are marked by rectangles. The numbers indicate the percentages of gated cells in the corresponding rectangle.

(C) As in (B), but mice were left untreated. BrdU was added to bone-marrow cells that were cultured for 1 hr in vitro in the absence of IL-7. Similar results were obtained after 1 day culture in vitro (not shown).

(D) μHC versus S27 FACS profiles of B220-gated cells are shown. Numbers show the percentages of cells in the indicated regions.

(E) RT-PCR analysis of 3-83Igi cells that were sorted for lack of BCR expression (BCR^-) or low BCR expression (BCR^{low}) as indicated. Data are representative of more than three independent experiments.

feature of the receptors in our cellular system and combined the HC of every polyreactive receptor with different LCs. We included an additional LC (LC40), which was derived from an antibody (eiJB40) with intermediate polyreactivity (Wardemann et al., 2004). The responsive BCR115 displayed a relatively high amount of receptor expression on the surface when combined with LC53 or LC62 as compared to surface expression with its original LC, whereas LC40 had an intermediate effect on receptor expression (Figure 2B). In addition, Ca^{2+} signaling upon induction of SLP-65 by OHT treatment was completely abrogated when the HC of BCR115 was combined with LC62, whereas variable Ca^{2+} flux was induced when LC53 or LC40 was used (Figure 2B). Furthermore, there was no enrichment of cells expressing HC115 in combination with LC53 and LC62, whereas LC40 resulted in intermediate enrichment (Figure 2B). In contrast to HC115, the refractory HC62 showed low levels of surface expression with all coexpressed LCs, of which LC40 showed the maximum reduction of receptor expression (Figure 2C). Moreover, cells expressing HC62 displayed efficient Ca^{2+} mobilization and enrichment irrespective of the coexpressed LC (Figure 2C). These data show that autonomous signaling (i.e., Ca^{2+} mobilization) of HC115, but not of HC62, can be inhibited by LC exchange, thereby preventing the relative enrichment of the respective cells.

Active Cycling of B Cells Expressing an Autoreactive Transgenic Receptor

Early B cells from 3-83Igi mice carry pre-rearranged 3-83 heavy- and light-chain V regions as transgenes in their physiological gene loci. The resulting 3-83 BCR recognizes MHC class I haplotypes with variable affinities (Pelanda et al., 1997). To test whether autoreactive BCRs induce proliferation and selection of early B cells in vivo, we first used the 3-83Igi mice on the $H\text{-}2K^b$ background, which is recognized with high affinity by the 3-83 BCR. According to the above results, constitutive BCR activation results in reduced BCR expression. Indeed, the bone marrow of 3-83Igi mice contained a distinguishable B cell population that showed low 3-83 LC (3-83κi) expression as detected by the antibody S27 (Figure 3A), which specifically recognizes 3-83κi (Casellas et al., 2001). Injection of 5′ -bromo-2′-deoxyuridine (BrdU) into 3-83Igi mice to assess cell-cycle activity in vivo showed that the highest BrdU incorporation is detected in B cells with low 3-83κi expression (Figure 3B, R1). However, B cells that lack the 3-83κi, and thus the autoreactivity, showed a substantial decrease in cell-cycle activity (Figure 3B, R2). Some B cells from wild-type (WT) mice express endogenous Vk4/5 LCs, which correspond to 3-83κi and can therefore be recognized by S27. Interestingly, S27-positive WT B cells showed reduced cell-cycle activity as compared with S27-negative B cells, which include SLC-expressing pre-B cells (Figure 3B and data not shown). To test whether the extensive in vivo cell cycling of autoreactive B cells is independent of IL-7, we performed BrdU incorporation experiments with bone marrow cells cultured for 1 hr or 24 hr in vitro in the absence of IL-7. In complete agreement with the in vivo data, the 3-83κi-positive B cells showed the highest cell-cycle activity, whereas cells that lack 3-83κi showed only minor cell cycling (Figure 3C, R1 and R2 and data not shown). In contrast to 3-83Igi B cells, the highest cell-cycle activity in culture was detected in R2, which includes conventional pre-B cells (Figure 3C, R1 and R2). After 2 days of in vitro culture, 3-83Igi B cells with low BCR expression were clearly enriched, whereas WT culture consisted mainly of BCR-negative B cells (Figure 3D).

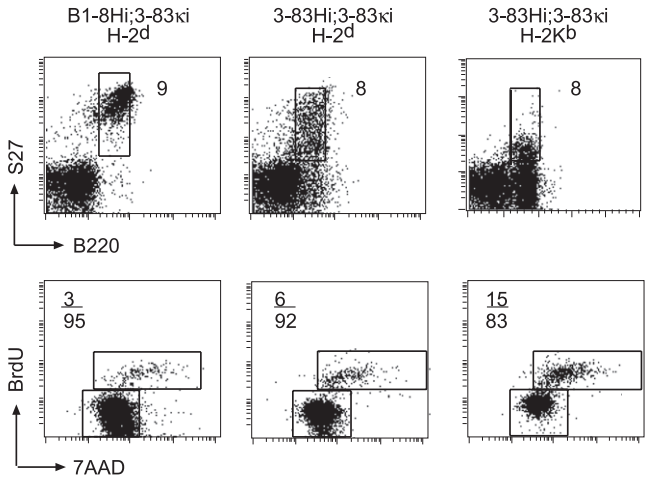

Figure 4. Increased Cell Cycling Correlates with High Self-Reactivity
B220 versus S27 (intracellular) and BrdU versus 7AAD FACS analysis of bone-marrow cells from the indicated mice. B1-8Hi,3-83κi mice express an unrelated knockin HC (B1-8Hi) together with the 3-83 LC. 3-83Hi,3-83κi indicates the HC and LC transgenes of the 3-83 BCR. The haplotypes of the MHCI background, H-2^d or H-$2K^b$, are shown. Recirculating cells were excluded by staining for CD23 (not shown). The numbers in the upper row indicate the percentages of the gated cells in the bone marrow. The numbers in the lower row indicate the percentages of the gated cells in the corresponding rectangle. Representative data of more than three independent experiments are shown.

To show that enrichment of the 3-83Igi B cells is independent of pre-BCR expression, we analyzed SLC expression in 3-83Igi B cells. RT-PCR analysis of sorted cells showed that BCR^{low} 3-83Igi B cells, which are the main cycling population, downregulate λ5 expression, suggesting that their increased cell-cycle activity does not require SLC expression (Figure 3E). Together, our experiments clearly indicate that autoreactive B cells proliferate in vivo and that this proliferation is independent of pre-BCR expression and IL-7. Of note, the 3-83Igi mice possessed WT SLP-65 gene loci, demonstrating that the enrichment of B cells expressing autoreactive BCRs occurs in the presence of SLP-65.

To characterize the developmental stage of the proliferating 3-83Igi B cells, we performed FACS analysis for early B cell markers such as c-kit, CD43, and CD25. We found that the proliferating 3-83κi-expressing cells were negative for c-kit and CD43, whereas a small fraction expressed CD25 (Figure S5 and data not shown). This suggests that the proliferating cells correspond to a developmental stage that physiologically expresses the BCR.

Previous results suggested that the 3-83 BCR is moderately self-reactive on the $H2^d$ background, whereas combining the 3-83κi with the unrelated B1-8 HC knock-in (B1-8Hi) creates a completely non-self-reactive BCR (Pelanda et al., 1997). Hence, we addressed the correlation between variable self-reactivity and the proliferation rate of autoreactive B cells. Our experiments show a low proliferation rate of B cells expressing a non-self-reactive BCR (B1-8Hi;3-83κi; H-2^d), whereas moderate proliferation rate was observed when the 3-83 BCR was expressed on the $H2^d$ background (3-83Hi;3-83κi; H-2^d). However, the highest proliferation rate was detected on the H-$2K^b$ background, in which the 3-83 BCR is highly self-reactive (3-83Hi;3-83κi; H-$2K^b$) (Figure 4). These results support the correlation between self-recognition and the proliferation rate of autoreactive B cells.

Secreted Pre-BCR Is Polyreactive

The efficient enrichment conferred by polyreactive BCRs raises the question as to how cells with nonpolyreactive HCs can be enriched in the bone marrow. Because pre-BCR expression precedes recombination of the LC gene locus and subsequent BCR expression, expansion of pre-B cells with successfully recombined HC genes is most likely to take place independently of the polyreactivity of the resulting HC. Therefore, we tested the ability of the different chimeric HCs to induce pre-B cell enrichment when combined with murine surrogate LCs (SLCs). We found that all HCs tested can pair with the SLC and thus form a pre-BCR displaying the characteristic low amount of receptor expression. In addition, all cells expressing a pre-BCR enriched over time, irrespective of whether their HCs were derived from a polyreactive BCR or not (Figure S6).

The surprising similarity between polyreactive BCRs and pre-BCRs prompted us to ask whether association of a nonpolyreactive HC with the SLC would turn the resulting pre-BCR into an autoreactive receptor, which is then capable of recognizing multiple antigens. We therefore expressed the SLC components VpreB1 and λ5 together with the B1-8 HC as well as with HC53 of the nonpolyreactive antibody mGO53 (Wardemann et al., 2004) and performed ELISA studies on well-defined antigens frequently used to analyze antibody poly- and autoreactivity. We observed that coexpression of B1-8 HC and HC53 with the SLC allowed efficient binding to all antigens tested. This binding was similar (on single- or double-stranded DNA) as or stronger (on insulin or LPS) than binding of eiJB40, for which intermediate polyreactivity has been described (Wardemann et al., 2004). Importantly, binding was strictly dependent on the λ5 non-Ig region because its deletion almost completely abolished binding (Figure 5). Association of the B1-8 HC or HC53 with their respective original LCs (B1-8LC and mGO53LC) did not lead to antigen binding. We have confirmed the association of both HCs with the SLC containing λ5 or λ5 lacking its non-Ig region by immunoblotting (data not shown). These data indicate that expression of a nonpolyreactive HC with the SLC turns the resulting pre-BCR into an autoreactive receptor capable of binding to multiple antigens accompanied by constitutive receptor signaling.

Redundancy between CDR3 and Non-Ig Region of λ5

The observation that polyreactive HCs led to enrichment with both conventional and surrogate LCs, whereas nonpolyreactive HCs needed the surrogate LC for enrichment suggests that polyreactive HCs may bear structural features that can replace the SLC in promoting signaling and growth advantage. The non-Ig region of λ5 has been demonstrated to be essential for pre-BCR function and is characterized by the occurrence of multiple arginine residues (Ohnishi and Melchers, 2003). Interestingly, polyreactive antibodies are often characterized by long complementarity-determining regions 3 (CDR3) enriched in positively charged (i.e., arginine) amino acids (Crouzier et al., 1995; Ichiyoshi and Casali, 1994; Koralov et al., 2006; Zhang et al.,

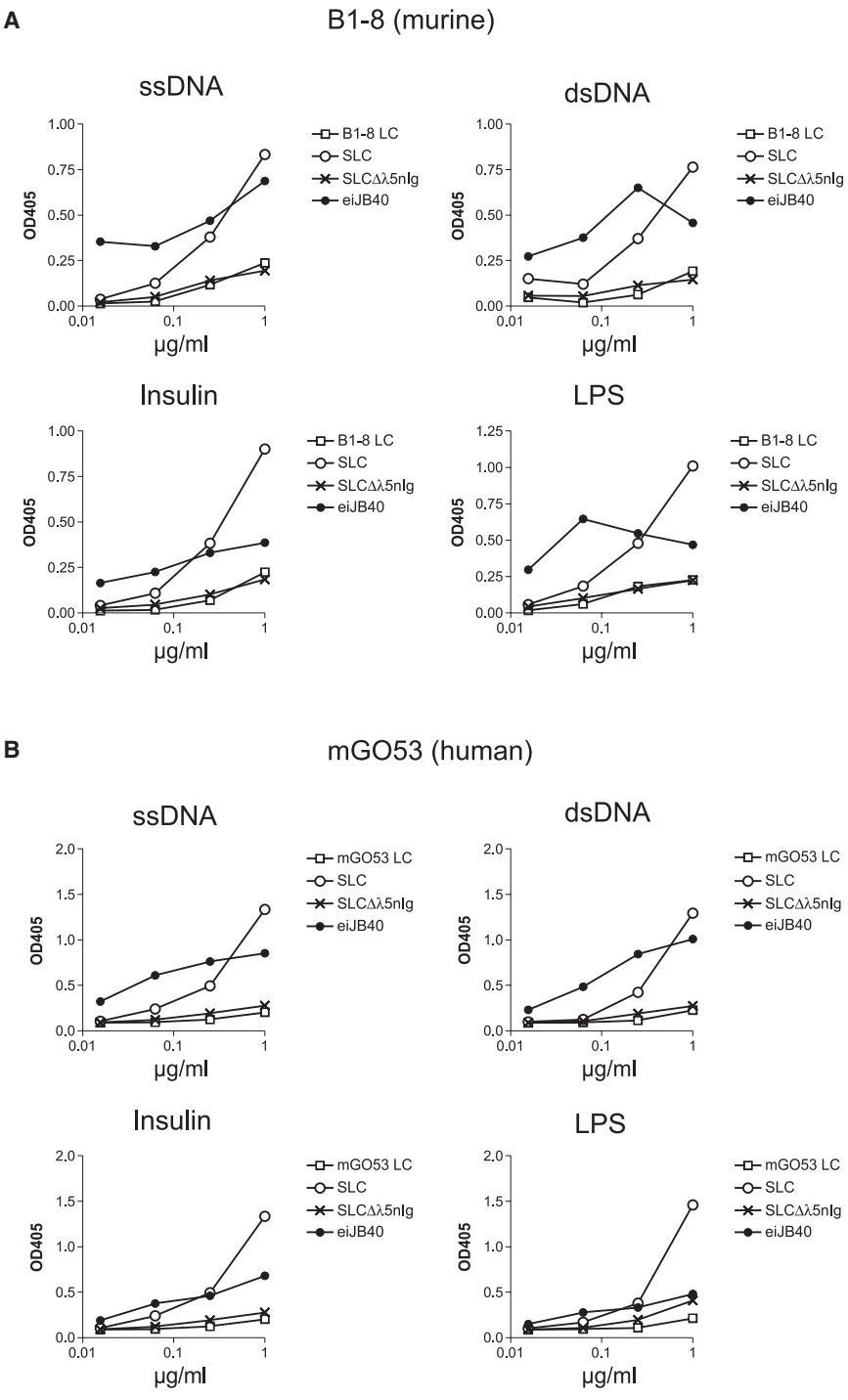

Figure 5. The SLC Confers Polyreactivity to Nonpolyreactive HCs

(A) ELISA showing binding to different antigens of a pre-BCR composed of SLC and the nonpolyreactive HC of murine B1-8. B1-8HC was combined either with its original LC (B1-8 LC), mSLC, or mSLC lacking the λ5 non-Ig region. The intermediately polyreactive eiJB40 served as control.

(B) As in (A), but with the HC of the nonpolyreactive human mGO53. Recombinant antibodies used in ELISA were IgG isotype. Representative data of three independent experiments are shown.

2004; Zhang et al., 2003); such a characterization is true for the polyreactive HC62 and HC115 but not for the nonpolyreactive HC53 (Figure S7). Therefore, we tested whether replacing the CDR3 of the nonpolyreactive HC53 by the λ5 non-Ig region would turn mGO53 into a polyreactive antibody, even when associated with its original LC and not with the SLC. We performed ELISA studies and observed very strong binding to all antigens tested, which was even more pronounced than with the highly polyreactive eiBC62 (Figure S7). Binding was observed when detected via both the LC (κ) or the HC (γ), showing that the antibodies bound were intact (Figure S8A). Secreted pre-BCR did not bind to control plates coated only with BSA, suggesting that binding to the above-mentioned antigens was not simply caused by SLC-mediated aggregation (Figure S8B). These data show that the non-Ig portion of λ5 can transfer polyreactivity when replacing the CDR3 of a nonpolyreactive antibody. To analyze whether determinants conferring polyreactivity can indeed be located either in the λ5 non-Ig region or in HC CDR3s, we tested the reciprocal situation, i.e., whether CDR3s of polyreactive antibodies can replace the non-Ig region of λ5. We compared the surface expression of mouse μHC paired with λ5, λ5Δnon-Ig (λ5U), or with λ5 in which the non-Ig region has been replaced by the CDR3s of HC53, HC62, or HC115 (λ5U-CDR3-53, (λ5U-CDR3-62 or (λ5U-CDR3-115) (Figure S9). As previously reported (Ohnishi and Melchers, 2003), cells expressing λ5U displayed high amounts of receptor surface expression as compared to λ5-expressing cells. When the λ5 non-Ig region was replaced by the CDR3 of the nonpolyreactive BCR53, surface expression was comparable to λ5U. Strikingly, when replaced by the CDR3s of the polyreactive HC62 or HC115, surface expression was reduced to amounts comparable to λ5-containing receptors (Figure 6). In addition, Ca^{2+} signaling was severely reduced when the λ5 non-Ig region was deleted and was only partially restored by replacement with the CDR3 of HC53. However, when the λ5 non-Ig region was substituted by the CDR3s of the polyreactive HC62 or HC115, Ca^{2+} signaling was restored to levels comparable with wild-type λ5. All of these variants displayed efficient Ca^{2+} mobilization when crosslinked by μ-chain antibody (Figure S10). Furthermore, enrichment of cells expressing λ5U or λ5U-CDR3-53 was greatly reduced as compared with λ5-expressing cells. In contrast, cells expressing λ5U-CDR3-62

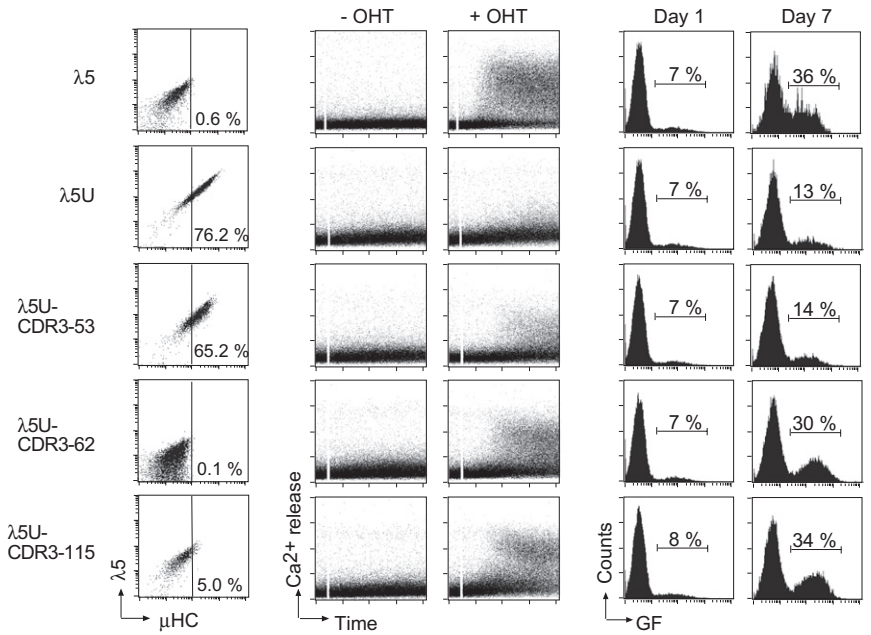

Figure 6. Arginine-Rich CDR3s of Polyreactive HCs Replaces the Non-Ig Region of λ5

μHC versus λ5 FACS analysis for receptor expression of triple-deficient cells expressing ERT2-SLP-65 and B1-8HC together with the indicated λ5-variants (left). Cells were transduced with the respective vectors depicted in Figure S9 and analyzed at day 1 after transduction for receptor surface expression. The numbers indicate the percentages of μHC high-expressing cells. Cells positive for ERT2-SLP-65 and respective receptors were subjected to Ca^{2+} measurement without or with the addition of 1 μM OHT (+OHT). Right panels show the relative increase of cells transduced with murine B1-8 μHC and the respective λ5 variants. The percentages of GF-positive cells were determined on day 1 and day 7 after transduction. Representative data of more than five independent experiments are shown.

or λ5U-CDR3-115 displayed efficient enrichment comparable with λ5-expressing cells (Figure 6). To test whether positively charged amino acids present in the CDR3s of the polyreactive HCs were involved in the restoration of λ5U function, we mutated the two arginines of CDR3-115 to glutamates. We observed an increase in surface expression of this mutant (Figure 7A) and a pronounced reduction in Ca^{2+} signaling (Figure 7B), although this signaling was stronger than with λ5U lacking the entire non-Ig region. Whereas these data are in agreement with an important role of positive charges in CDR3s of polyreactive HCs, they suggest a potential role for the residual amino acids of the CDR3-115 in the restoration of λ5U function.

DISCUSSION

We have described an unexpected functional similarity between autoreactive BCRs and the pre-BCR suggesting that recognition of self-antigens might play an essential role in the positive selection of early B cells. Our data allow us to draw three important conclusions, which are essential for understanding early B cell development. First, because the pre-BCR and autoreactive BCRs are functionally equivalent, early B cells may not discriminate between auto-crosslinked pre-BCRs and antigen-crosslinked autoreactive BCRs. Second, because both the pre-BCR and autoreactive BCRs induce expansion and positive selection, it is likely that the elevated proportion of autoreactive B cells in the bone marrow is the result of increased survival and expansion of the corresponding cells (Wardemann et al., 2003). Third, receptor editing by secondary IgL-gene rearrangements might not represent an independent process, but rather recapitulates pre-BCR signaling that induces IgL gene recombination. The only difference would be that the pre-BCR induces primary, whereas autoreactive BCRs induce secondary LC recombination (Gay et al., 1993; Melamed et al., 1998; Melamed and Nemazee, 1997; Nemazee, 1996; Prak and Weigert, 1995).

The functional mimicry between the pre-BCR and polyreactive BCRs suggests that the pre-BCR can undergo low-affinity recognition of multiple self-antigens. The polyreactive potential and the variable structures present in each pre-BCR may well lead to direct ligand-independent interactions between pre-BCR complexes (Bankovich et al., 2007; Ohnishi and Melchers, 2003). Such mutual recognition of pre-BCR complexes independent of additional structures might be kinetically preferred, given that pre-BCR complexes are formed in a timely and spatially close relation. As for the pre-BCR, polyreactive BCRs may also bind directly to one another independently of additional antigens by recognizing structures that are present in the receptors themselves. On the other hand, the polyreactivity of the pre-BCR might explain reports describing pre-BCR interaction with distinct structures. Thus, the described ligands galectin-1 (Gauthier et al., 2002) or heparan sulfates (Bradl et al., 2003) may interact with the pre-BCR because of the polyreactive capacity of the latter. Another potential structure that might be recognized by the pre-BCR or polyreactive BCRs is DNA because the presence of arginine residues is a common feature of DNA-binding Ab and adding arginine residues to DNA-binding V regions increases the affinity for DNA and the tendency to receptor editing (Li et al., 2001; Radic et al., 1993; Radic and Weigert, 1994; Shlomchik et al., 1990; Shlomchik et al., 1987).

Compared with conventional transgenic approaches, 3-83Igi transgenic mice provide a physiologically optimal system to study the role of autoreactive BCRs in early B cell development in vivo. Interestingly, the phenotype of the 3-83Igi mice carrying targeted insertions of rearranged IgH and IgL genes encoding an autoreactive BCR pointed to a functional similarity between autoreactive BCRs and the pre-BCR (Pelanda et al., 1997). Because normal B cell development and a rather increased population of large pre-B cells were observed in autoreactive 3-83Igi mice, whereas their nonautoreactive counterparts showed a drastically reduced pre-B cell compartment, it was suggested that the pre-BCR directs progenitor B cells into the editing

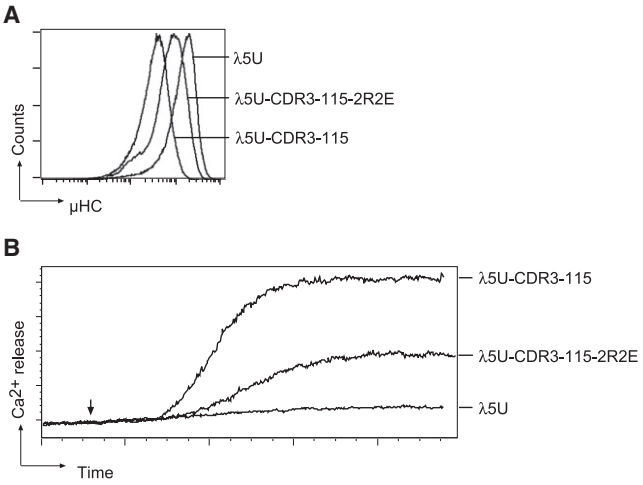

Figure 7. Positive Charges in the CDR3 Are Important for the Functional Redundancy

(A) Surface expression and (B) Ca^{2+} measurement upon OHT-treatment of triple-deficient cells expressing inducible SLP-65 and B1-8 μHC in combination with different λ5 variants. The variants include λ5 in which the non-Ig region was deleted (λ5U) or replaced either by the HC-CDR3 of the polyreactive BCR115 (λ5U-CDR3-115) or by a mutant thereof in which the two arginines have been changed to glutamates (λ5U-CDR3-115-2R2E). Representative data of more than three independent experiments are shown.

pathway by mimicking autoreactive BCRs (Pelanda et al., 1997). This view is reminiscent of a previous concept that suggested that the selection pressure for initiating the generation of immune cells must be exerted by the antigens of the animal itself (Jerne, 2004). Our data are in complete accordance with this view, in that they show that 3-83Igi B cells have a high amount of cell-cycle activity as long as they express the autoreactive BCR specificity. Thus, the increased cell cycling is critically dependent on the expression of the autoreactive BCR, but not on pre-BCR expression because cycling 3-83Igi B cells downregulate λ5 expression. Interestingly, the downregulation of λ5 in 3-83Igi B cells is most likely a further result of the functional similarity between autoreactive BCRs and the pre-BCR, which usually induces λ5 downregulation (Meixlsperger et al., 2007; Thompson et al., 2007). By identifying the functional similarity between the CDR3 regions of autoreactive μHCs and the non-Ig region of λ5, our data describe the molecular mechanism, which enables autoreactive BCRs to function like the pre-BCR.

Although it is generally accepted that BCR expression is an absolute requirement for the development and survival of B cells (Lam et al., 1997; Torres et al., 1996), the role of self-Ag in positive selection of B cells remains unclear. Available data suggest that polyreactive low-affinity BCRs are involved in the generation and maintenance of B-1 B cells, which produce primarily autoreactive antibodies. These so-called "natural autoantibodies" represent a large fraction of serum Ig in healthy individuals and play an important role in protection from bacterial infections (Hardy, 2006). The positive role of self-Ag in selecting B-1 cells was clearly demonstrated by mice transgenic for a naturally generated autoreactive BCR that recognizes Thy-1, a glycoprotein expressed on T cells (Hayakawa et al., 1999). These experiments showed that anti-Thy-1-specific B cells do not accumulate in antigen-deficient (Thy-$1^{-/-}$) mice. A positive role of self-Ag for the selection of B cells has also been demonstrated by transgenic mice expressing a low-affinity polyreactive BCR recognizing ssDNA, thyroglobulin, human Fcγ, and phosphatidylcholine (Julien et al., 2002; Wang and Clarke, 2003; Wang and Clarke, 2004). Thus, increasing evidence suggests that autoreactivity and recognition of self-Ag may well be involved in the positive selection of developing B cells.

Interestingly, recent data with SLC transgenic mice, which continue to express the pre-BCR beyond the pre-B cell stage, show (1) a severe developmental block and (2) substantial amount of secondary rearrangement of the LC gene loci (van Loo et al., 2007). Because both are characteristic features of mice expressing transgenes for autoreactive receptors, the SLC transgenic mice provide further support for the functional similarity between the pre-BCR and autoreactive BCRs.

Together, our results suggest that the pre-BCR is most likely the prototype of an autoreactive receptor with a timely restricted SLC expression to ensure that autonomous signaling is strictly controlled. In light of our results, it is tempting to speculate that during evolution the mechanism of expanding the pool of B cell precursors, which have undergone successful HC recombination, may have developed from being based on polyreactive HCs with long and positively charged CDR3s to a mechanism involving the timely restricted expression of a λ5-containing SLC. Interestingly, as we show here for the pre-BCR, in pre-T cell receptors (pre-TCRs), positive charges in the extracellular part of pTα have also been implicated in proliferation and signaling via the pre-TCR, allowing for efficient T cell development and β chain selection (Yamasaki et al., 2006). Thus, it is an appealing idea that analogous to the pre-BCR, the pre-TCR may be crosslinked by different (self-) antigens.

EXPERIMENTAL PROCEDURES

Mice

The generation of 3-83Igi mice carrying the pre-rearranged Ig gene segments was described previously (Pelanda et al., 1997). The expression of H-2^d and H-$2K^b$ was determined by specific monoclonal antibodies (BD PharMingen). For BrdU-labeling experiments, mice were injected with 100 μl of 10 mg/ml stock solution. After 1 hr, bone-marrow cells were analyzed for incorporation of BrdU and for DNA content (7AAD staining) with the BrdU Flow Kit (BD PharMingen). Animal experiments were done in compliance with guidelines of German law and the MPI for Immunobiology.

Cells and Culture Conditions

Triple-deficient cells (Meixlsperger et al., 2007) were cultured in Iscove's medium containing 10% heat-inactivated fetal-calf serum (Vitromex), 100 U/ml of penicillin, 100 U/ml of streptomycin (GIBCO) and 5×10^{-5} M 2-mercaptoethanol. Supernatant of J558L cells stably transfected with a vector for murine IL-7 was added. BrdU labeling was performed with the BrdU Flow Kit according to the manufacturer's instructions (BD PharMingen).

Retroviral Constructs and Transductions

ERT2-SLP-65 (Meixlsperger et al., 2007) and SLP-65 were expressed from retroviral vectors encompassing tdTomato (Shaner et al., 2004) (kind gift from R.Y. Tsien) as an IRES cassette. IgHCs were expressed from retroviral vectors encompassing a fusion of the yeast GCN4 leucine zipper and a C-terminal fragment of CFP, whereas expression vectors for IgLCs included the leucine zipper and an N-terminal fragment of YFP. Human-mouse chimeric HCs were generated by PCR-amplification of human IgH VDJ regions and ligation to the murine μJ558L HC constant region. We generated λ5U-CDR3 constructs by replacing the λ5non-Ig region by the CDR3s of the respective

HCs. We constructed the mGO53H-CDR3/λ5nIg by replacing the HC CDR3 by the λ5 non-Ig region. The λ5U-CDR3-115-2R2E mutant was generated through directed mutagenesis by sequential PCR steps. Detailed description of the cloning strategies is available upon request. Retroviral transductions were essentially done as described (Su and Jumaa, 2003).

Ca^{2+} Measurements

Ca^{2+} measurements were performed as described (Storch et al., 2007). A total of 5×10^6 cells were incubated with 5 μg/mL of Indo-1 (Molecular Probes) and 0.5 μg/mL of pluronic F-127 (Molecular Probes) in Iscove's medium supplemented with 1% FCS (Vitromex) at 37°C for 45 min. The cell pellet was resuspended in Iscove's medium with 1% FCS, and calcium response was induced by adding 10 μg/mL of goat anti-mouse IgM (Southern Biotechnology) or 1 μM of OHT. Calcium flux was measured with the LSRII (Becton Dickinson).

Flow Cytometry

Cells were stained for FACS with anti-IgM-Cy5 (μHC specific, Dianova), anti-λ-biotin (Biozol), anti-λ5 (LM34-biotin) (Karasuyama et al., 1993), and Streptavidin-PE (Jackson ImmunoResearch). Human LCs were stained with anti-λ (LC115, Southern Biotech) or anti-κ (LC40/53/62, Southern Biotech). S27, the antibody recognizing the 3-83 LC (3-83κi), was kindly provided by J. Kirberg. ERT2-SLP-65 was detected directly via tdTomato (Shaner et al., 2004). FACS analysis was performed with FACSCalibur (Becton Dickinson).

Enrichment Studies

Determination of cell increase was carried out in medium containing limiting concentrations of murine recombinant IL-7 (Sigma), and the percentages of GF-positive cells were measured by FACS analysis at different time points after transduction.

Antibody Production and ELISA Studies

Antibody production and ELISA studies were performed as described (Wardemann et al., 2003). 293A human embryonic kidney fibroblasts were cultured in DMEM supplemented with 10% ultra-low IgG FCS (GIBCO) and cotransfected with 12.5 μg of IgH and IgL chain encoding plasmid DNA by calcium phosphate precipitation. Eight to twelve hours after transfection, cells were washed with serum-free DMEM and thereafter cultured in DMEM supplemented with 1% Nutridoma SP (Roche). Supernatants were collected after 8 days of culture and purified on protein G Sepharose (Amersham Pharmacia Biosciences). For reactivity with specific antigens, microtiterplates (COSTAR Easywash Polystyrene Plates, Corning) were coated with 10 μg/ml of ssDNA, dsDNA, or LPS (Sigma) or 5 μg/ml recombinant human insulin (Fitzgerald). Tissue-culture supernatants were used at 1 μg/ml antibody concentrations and three 1:4 dilutions in PBS. All ELISAs were developed with HRP-labeled goat anti-human IgG Fc Ab (Jackson ImmunoReseach) and HRP Substrate (BIO-RAD). OD405 was measured using a microplate reader (Molecular Devices).

SUPPLEMENTAL DATA

Supplemental Data include ten figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(08)00505-0.

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