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Multiple Levels of Selection Responsive to Immunoglobulin Light Chain and Heavy Chain Structures Impede the Development of Dμ-Expressing B Cells[1]

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The truncated/V_H-less mouse H chain Dμ forms precursor B cell receptors with the surrogate L chain complex that promotes allelic exclusion but not other aspects of pre-B cell development, causing most progenitor B cells expressing this H chain to be eliminated at the pre-B cell checkpoint. However, there is evidence that Dμ-λ1 complexes can be made and are positively selected during fetal life but cannot sustain adult B lymphopoiesis. How surrogate and conventional L chains interpret Dμ's unusual structure and how that affects signaling outcome are unclear. Using nonlymphoid and primary mouse B cells, we show that secretion-competent λ1 L chains could associate with both full-length H chains and Dμ, whereas secretion-incompetent λ1 L chains could only do so with full-length H chains. In contrast, Dμ could not form receptors with a panel of κ L chains irrespective of their secretion properties. This was due to an incompatibility of Dμ with the κ-joining and constant regions. Finally, the Dμ-λ1 receptor was less active than the full-length mouse μ-λ1 receptor in promoting growth under conditions of limiting IL-7. Thus, multiple receptor-dependent mechanisms operating at all stages of B cell development limit the contribution of B cells with Dμ H chain alleles to the repertoire. ***The Journal of Immunology,*** **2008, 181: 4098–4106.**

B cell development depends on the expression of structurally sound Ig H chain and L chain proteins (reviewed in Ref. 1). H chain and L chain proteins form signal transduction complexes that are required to activate programs of differentiation and cell growth. These receptors thereby serve as a quality control mechanism to establish whether the V(D)J rearrangement process, necessary to assemble Ig genes from component gene segments, created a functional Ig gene. For example, before L chain rearrangement, progression from the progenitor (pro) to precursor (pre) stage of development depends on the ability of the H chain to form a so called precursor B cell receptor (preBCR)[4] complex with the surrogate L chain (SLC) components λ5 and VpreB and signal transducers Igα and Igβ (2–4). B cells that synthesize no H chains or H chains that fail to form signaling-competent receptor complexes (either with or without the SLC) due to intrinsic structural flaws are eliminated because of the absence of a preBCR signal. In this way, the SLC selects for H chains with the best likelihood of forming BCRs with L chains that can be regulated by Ag (5, 6).

Other SLC-dependent mechanisms prevent the emergence of B cells expressing Dμ, a truncated mouse H chain that lacks a V_H region (Ref. 7; reviewed in Ref. 8). Dμ can be synthesized in the mouse before V_H-to-DJ_H joining when D_H and J_H are joined using reading frame 2 (RF2) of D_H. If Dμ were innocuous, more than half of all B cells should carry at least one such rearrangement; however, D_H-J_H and V_H-DJ_H rearrangements using D_H RF2 are vastly underrepresented as early as the pre-B and later mature B cell stages (9, 10). This is because Dμ associates with the SLC complex to form an active but defective preBCR (reviewed in Ref.11). Studies in vivo with Dμ-transgenic mice have shown that Dμ, like most SLC-dependent full-length H chains, can enact allelic exclusion (suppress V_H-to-DJ_H rearrangement), but in contrast it signals poorly if at all for survival, proliferation, or differentiation of pro-B cells to small pre-B cells and possibly later stages (12, 13). Thus, pro-B cells expressing Dμ could neither developmentally progress nor continue IgH recombination to replace the Dμ rearrangement. A molecular correlate to the signaling impairment is that Dμ-preBCRs fail to be transported out of the endoplasmic reticulum (ER) to reach post-ER compartments and the cell surface as efficiently as normal preBCRs with full-length H chains (14–18). Mutational analysis of the SLC demonstrated this was in part because VpreB requires a V_H partner for this to occur optimally (16).

There is also indication that L chain-dependent counterselective mechanisms exist at later developmental stages to block the emergence of $D\mu^+$ B cells. Hypothetically, L chain partners that could accommodate Dμ's unusual structure and form receptors with it might be able to allow Dμ signaling and promote the emergence of B cells with Dμ alleles. However, there is still a bias against RF2 in mature B cells of λ5-deficient mice (19). To help explain this, biochemical studies have shown that Dμ could not associate with two representative κ L chains (15, 17), which was taken to suggest that Dμ-κ complexes could not be made. If this were categorically

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[4] Abbreviations used in this paper: preBCR, precursor B cell receptor; ER, endoplasmic reticulum; HEK, human embryonic kidney; IRES, internal ribosomal entry site; pre-B, precursor B; pro-B, progenitor B; RF2, reading frame 2; SLC, surrogate light chain; UR, unique region.

true, given that κ is the favored L chain isotype in mice, this would be a major barrier for emergence of Dμ-containing B cells.

However, in vivo there is positive selection of in-frame λ1 rearrangements in SCID mice, but not in Rag-deficient mice, which has been attributed to the expression of Dμ H chains (20). Although in SCID mice V_H-to-DJ_H recombination is profoundly impaired, productive λ1 and D_H-to-J_H rearrangements occur and thus potentially produce Dμ-λ1 receptors responsible for that selection (20). In support of this model, we have shown that Dμ could form Dμ-λ1 receptors that drove proliferation and differentiation of pro-B cells in some cases as well as full-length μ-λ1 complexes (17). Even though in mice λ L chain genes are rearranged later during the pre-B cell stage and less frequently than κ (21), this level is sufficient to allow positive selection of $IgM^+λ1^+$ B cells in κ L chain-deficient mice (22, 23). Nevertheless, mature B cells expressing exclusively Dμ-λ1 have not been reported in the adult even when Dμ expression was enforced via a transgene (24), suggesting that other levels of counterselection sensitive to H chain structure might be responsible for preventing the appearance of Dμ-λ1 B cells.

The goals in this study were to determine what structural features and properties of λ and κ L chains would be required for Dμ receptor formation and to gain further insight as to how Dμ structure affects receptor activity. One objective was to establish the relationship of the ability of a L chain to fold autonomously and be secreted to pairing with Dμ. This is because in our studies and in those of others (15, 16), the representative λ1 L chain was secretion competent, whereas the κ L chains were not and depended on association with a full-length H chain for ER release and surface expression. It was hypothesized that this may be an important parameter limiting L chain/Dμ association because Dμ lacks a V_H region with which a V_L could fold and pair. Given that in several functional assays Dμ-λ1 and μ-λ1 receptors exhibited similar activity (17), another objective was to ask whether other, more stringent conditions might reveal Dμ-λ1 signaling impairments that may help explain why these receptors are not found in the adult. Herein, we show that secretion competency of λ1 L chains was a critical determinant controlling assembly of this class of L chains with Dμ. In contrast, the J and C regions of κ were the intrinsically prohibitive elements irrespective of κ L chain folding status. Studies in primary cells indicated that Dμ-containing receptor complexes were on average less active in supporting cell growth under conditions of limiting IL-7 than wild-type complexes. These results indicate that conventional L chains are in general structurally and functionally incompatible with Dμ. Thus, they serve in synergy with the SLC to block the emergence of B cells expressing this H chain first by severely limiting the frequency of Dμ-BCR formation and then by restricting the signaling output of any rare but fully assembled Dμ-BCR complexes.

Materials and Methods

Plasmids

The creation of cDNAs encoding the mouse μ H chain 17.2.25, Dμ, the human μ H chain TG.SA (T), λ1, MOPCκ, and JCκ have been described previously (16). Briefly, JCκ was made by replacing the leader and Vκ sequence of a MOPC21κ L chain with the leader of λ1. λ1–λ5 fusions were created by PCR mutagenesis. Secκ was created by replacing His^{87} in the V domain of MOPC21κ with Tyr by PCR mutagenesis. The V_L of MPC11κ was cloned from mouse genomic DNA using the published MPC11κ sequence (25) and fused to the JCκ region of MOPC21κ by PCR. The cDNAs were subcloned into either MiG (26), a murine retroviral construct that contains the gene encoding the marker GFP linked to the cDNA via an internal ribosomal entry site (IRES), or to MihCD4Δ, a retroviral plasmid that contains the marker gene encoding hCD4Δ similarly linked via an IRES. MihCD4Δ was created by replacing IRES-GFP in MiG with the IRES-hCD4Δ sequence from pMACS 4.1 (Miltenyi Biotec).

Cells and in vitro cell culture

Human embryonic kidney (HEK) epithelial 293 cells were grown in DMEM supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin, and L -glutamine (PSG). Short-term primary IL-7-dependent pro-B cell cultures were established by harvesting and plating total bone marrow of 4–6-wk-old $Rag1^{-/-}λ5^{-/-}$ mice (27, 28) in RPMI 1640 (Invitrogen) supplemented with 10% FBS, antibiotics (1% penicillin-streptomycin, L -glutamine), 5×10^{-5} M 2-ME, and recombinant IL-7 (100 U/ml = 5 ng/ml; Cell Sciences). Cells were seeded at a density of $0.5–2 \times 10^6$ cell/ml and maintained in culture for 2 days before retroviral infections (29, 30). The $Rag1^{-/-}λ5^{-/-}$ v-abl-transformed cells have been described (17). Mouse usage was reviewed and approved by the State University of New York–Downstate Medical Center Institutional Animal Care and Use Committee.

Transient transfections

HEK293 cells were cotransfected by calcium phosphate-mediated precipitation of MiG-L chain (4 μg), pEBB-H chain (4 μg), pEBB-Igβ (2 μg), and pEBB-Igα (2 μg) plasmids as described (16). Empty pEBB vector was used to normalize the total amount of DNA introduced to cells. Two days later, cells were harvested by incubation with PBS supplemented with 10 mM EDTA, followed by pipetting into single-cell suspensions. Aliquots were prepared for flow cytometry and Western blot analysis as described (16).

Retroviral infections

Retroviruses were produced by calcium phosphate-mediated cotransfection of HEK293 cells with retroviral plasmids plus pψECO, which encodes ecotropic helper functions (31). Primary cells were spin-infected with recovered supernatants as described (17). Briefly, viral supernatants and polybrene (4–8 μg/ml) were added to $0.5–2 \times 10^6$ bone marrow cells per well in 12- or 24-well plates, followed by centrifugation at 2500 rpm at 25°C for 1.5 h. Supernatants were then replaced with fresh medium supplemented with 100 U/ml IL-7 after infection. For double infections, the spin infection was repeated twice with 1 day between infections. Double infections of primary IL-7-dependent pro-B cells were done by first infecting cells with the L chain-MiG viruses, then splitting the infected cells into separate wells for infection with H chain viruses that did not contain a marker gene. Consequently, relative amounts of H chain expression between infected cultures were determined by Western blot of infected cells. Cells were analyzed 2–4 days after infection by flow cytometry and Western blot. Infections of v-abl-transformed cells were done as described (17).

Flow cytometry to track surface marker expression and cell growth

Single-cell suspensions were stained with the following Abs (directed against mouse Ags except where noted) for flow cytometry by standard protocols: anti-CD19-TRI and anti-mouse IgM-PE from Caltag Laboratories; anti-λ5-biotin (LM34), anti-CD2-PE, anti-CD22-PE, and streptavidin-PE from BD Pharmingen; and anti-human IgM-PE and anti-λ1-PE from SouthernBiotech. Analyses of CD2, CD22, and proliferation were performed as described (17, 18). Briefly, the values shown for CD2 and CD22 induction are the percentages of $CD19^+GFP^+$ cells that were also $CD2^+$ and $CD22^+$ 4 days and 2 days after infection, respectively. Relative growth was defined as the fold change in the percentge of $CD19^+GFP^+$ cells in cultures after 24 or 48 h divided by the fold change in the percentage of $CD19^+GFP^-$ cells in the same culture over the same time period. The fold change in $CD19^+GFP^-$ cells in each sample was defined as 1 (no change in relative growth rate) in the bar graphs.

Comparison of the relative abilities of preBCRs/BCRs to support growth over a concentration gradient of IL-7

Two days after infection, each sample was equally divided into six separate cultures, and each was subcultured in different concentrations of IL-7 in 10-fold dilutions from 100 U/ml (5 ng/ml) to 0.01 U/ml (0.5 pg/ml). After 4 days, the growth of $CD19^+GFP^+$ cells relative to the growth of $CD19^+GFP^-$ cells in each culture was calculated, as above. The numbers plotted in the bar graph in Fig. 5 were calculated by dividing the relative fold change in $CD19^+GFP^+$ cells at 100 or 0.1 U/ml IL-7 to the relative fold change in $CD19^+GFP^+$ cells in the 100 U/ml IL-7 cultures.

Western blotting

Western blots were performed as described (17). The following Abs were used for Western blot: rabbit and goat anti-mouse IgM, μ H chain-specific, hamster γ globulin from Jackson ImmunoResearch Laboratories, and goat

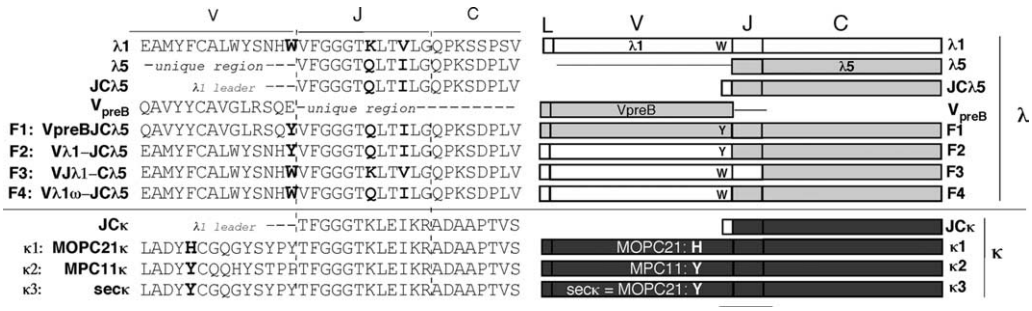

FIGURE 1. Ig L Chains used in this study. Shown on the left is an alignment of the amino acid sequences of the CDR3 region of the naturally occurring and engineered surrogate and conventional L chain molecules used in this study. The amino acids in boldface type in the V regions (Y and W, λ chains; H and Y, κ chains) are those targeted for site-directed mutation that are different among the matched pairs of λ (*top set*) or κ L chains (*bottom set*), as discussed in the text. Boldface amino acids in the λ J regions are naturally occurring but are different between λ1 and λ5 chains. Adjacent is a schematic of the natural and engineered L chains (leader (L), V, J, and C regions, not drawn to scale) with the underlined section designating the location of the amino acid sequences elaborated above.

anti-mouse κ and λ1 from SouthernBiotech. All AP- and HRP-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories and Caltag Laboratories.

Results

λ L chains must be secretion competent to form surface receptors with Dμ

Our previous studies showed that a secretion-competent λ1 L chain could associate and form surface-expressed, signaling-competent receptor complexes with Dμ, whereas a secretion-incompetent κ L chain did not associate with Dμ (16, 17). Similarly, JCλ5, a truncated λ5 molecule that lacks the UR and is secretion competent, and JCλ1, a secretion-competent and truncated λ1 chain, could associate with Dμ and support Dμ surface expression and, when tested, signaling (Refs. 16, 17 and data not shown). Given that Dμ lacks a V_H region, these results suggested that the particular Vκ and the VpreB and λ5UR components of the SLC required a complementary V_H domain from the H chain partner to fold properly and thereby to allow efficient release of the resultant receptor complexes from the ER. We therefore determined whether secretion competence was a key and general property of L chains indicative of their ability to form receptors with Dμ or if there were other structural features of κ L chains not related to secretory status that were determining factors.

The sequences and properties of the λ class of L chains (which includes the SLC) that controlled receptor formation with Dμ were determined by testing the ability of a panel of chimeric λ L chain-SLC fusion proteins constructed from VpreB, λ5, and λ1 chains to form receptors with Dμ and μ H chains. Fusion F1 (VpreBΔUR-JCλ5) was a single-chain version of the SLC that lacked the unique regions (URs) of both VpreB and λ5, also permitting the evaluation of the URs to receptor formation (Fig. 1). Fusions F2, F3, and F4 contained the JC or C regions of λ5 linked to the V_L of λ1 rather than to VpreB (Fig. 1). The ability of these fusion proteins to form receptors with Dμ or μ H chains was first tested in a nonlymphoid human embryonic kidney (HEK293) cell line. Igα- and Igβ-expression plasmids were cotransfected with those carrying the L chain and/or H chain genes to complete the receptor components (16).

F1:VpreBΔUR-JCλ5, F2:Vλ1-JCλ5, and F3:VJλ1-Cλ5 L chains were all comparable to λ1 in escorting μ to the cell surface of transfected cells (Fig. 2*A*, *top and bottom sets of panels*, and *B*, *top panel*; anti-IgM), indicating that these L chain fusion proteins were structurally sound and compatible with a normal H chain. On the other hand, only F3:VJλ1-Cλ5, but not F2:Vλ1-JCλ5 or F1:VpreBΔUR-JCλ5, could form a surface receptor complex with Dμ (Fig. 2*A*, *top set of panels*, and 2*B*; anti-IgM). Detection of surface complexes with λ1 and λ5 Abs concurred (Fig. 2*A*, *middle and bottom sets of panels*, and 2*B*, *middle and bottom panels*). Consistent with the flow cytometry data, Western blot analysis of extracts from transfected cells showed similar amounts of the mature, endo-H-resistant form of μ in the presence of λ1 or the λ1–λ5 and VpreBΔUR-JCλ5 fusion proteins (Fig. 2*C*). Similarly, the major fraction of Dμ was endo-H resistant in Dμ- F3:VJλ1-Cλ5- and Dμ-λ1-expressing cells, but dramatically lower to undetectable in Dμ-, Dμ plus F2:Vλ1-JCλ5-, or F1:VpreBΔUR-JCλ5-expressing cells (Fig. 2*C*). In parallel with Dμ receptor formation capability, F3:VJλ1-Cλ5 and λ1, but not F2:Vλ1-JCλ5 or F1:VpreBΔUR-JCλ5, were secreted into the medium, even though all of the L chains were expressed at comparable levels within the cells (F2 and F3, Fig. 2*D*; F1, data not shown). Therefore, the ability of these hybrid λ L chains to form surface receptors with Dμ directly correlated with their secretion competency in the nonlymphoid cells.

Only three amino acids that differ between F2:Vλ1-JCλ5 and F3:VJλ1-Cλ5 could account for their differences in secretion competency, with one lying at the junction of the V-J segments and two within the different Js (Fig. 1). Indeed, replacement of the F2 Tyr with Trp was sufficient to convert F2 into a secretion-competent L chain (F4; Figs. 1 and 2*D*). This new L chain, referred to as F4: Vλ1ω-JCλ5, behaved comparably to λ1 with respect to forming surface complexes with Dμ (Fig. 2). Therefore, for the λ class of L chains (λ1 and λ5), the barrier for Dμ receptor formation and surface expression appeared to be imposed by the folding properties of the V_L domain, either Vλ1 or VpreB.

We then asked whether these properties were also evident under more physiological conditions, namely, in primary, IL-7-dependent pro-B cells, which represent the first stage in B cell development in which Dμ and L chains could hypothetically be coexpressed. cDNAs encoding H chain and L chain receptor components were retrovirally transduced into H chain- and L chain-deficient $Rag1^{-/-}\lambda5^{-/-}$ pro-B cells, which express only the VpreB component of the SLC (17). We focused on comparing Dμ and μ complexes with secretion-incompetent F2 (nonsecreted; herein referred to as $F2^{NS}$) and F4 (secreted; $F4^{SEC}$) λ class L chains because only a single amino acid differs between them. Unlike HEK293 cells, the μ-$F2^{NS}$ receptors were expressed at >10-fold lower levels on the surface than were μ-$F4^{SEC}$ and μ-λ1 receptors (Fig. 3*A*, IgM; Fig. 3*B*, λ1). Western analysis of infected cells showed that this was not due to differences in total H chain expression but rather corresponded to a proportional decrease in

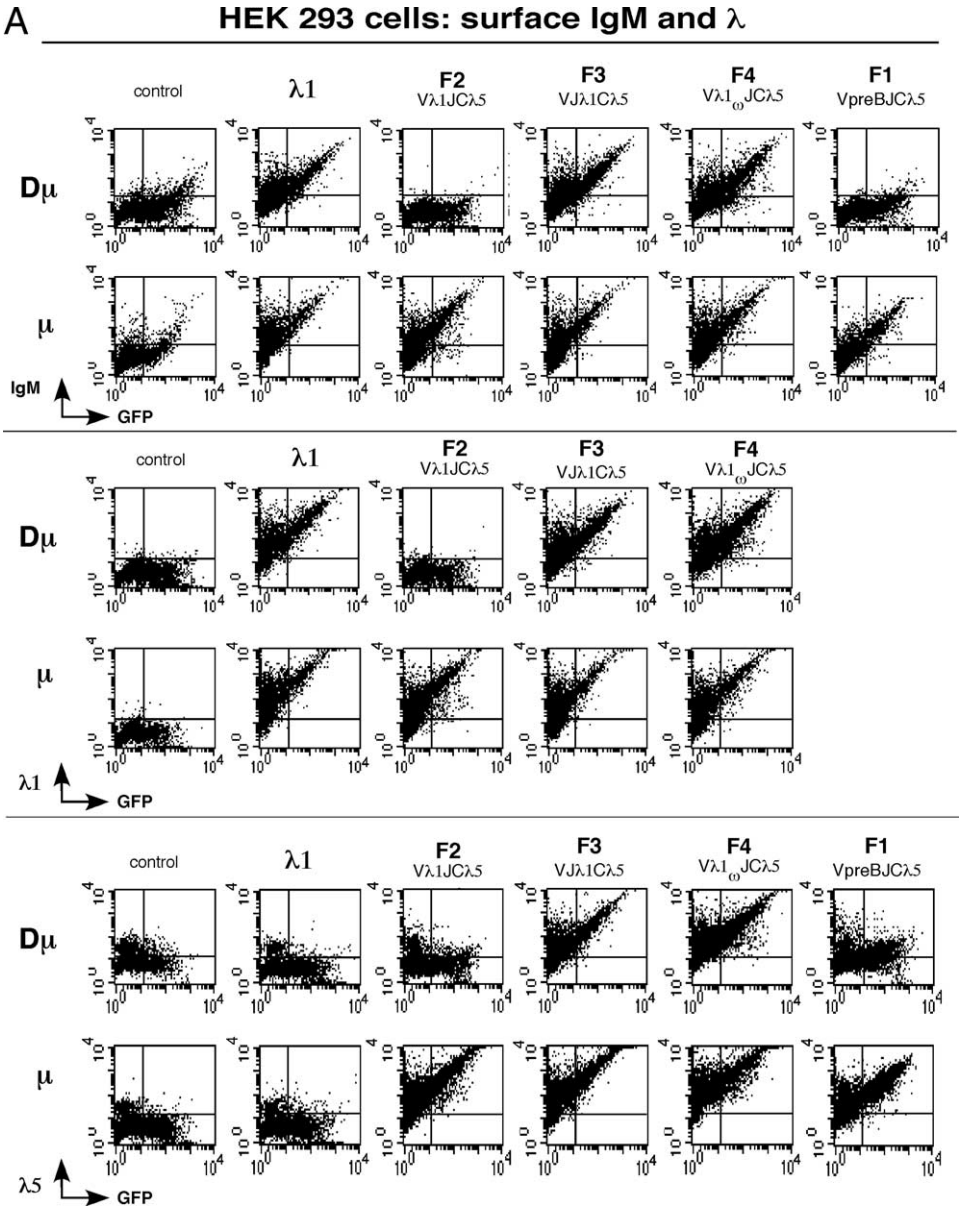

FIGURE 2. BCR formation and L chain secretion in nonlymphoid cells. *A*, Dot plots of surface IgM, λ1, and λ5 epitope expression (*y*-axis) by HEK293 cells cotransfected with the indicated H chain and L chain pairs. Transfected cells express the GFP protein (*x*-axis). *B*, Bar graph representation of surface stains plotting relative mean fluorescence from *A*. L chains are indicated below each bar; Dμ and mouse μ H chains are indicated in each panel. *C*, Western blot of Dμ and μ H chains in HEK293 cells cotransfected with the indicated H chain and L chain constructs. *Top panel*, Untreated extract; *bottom panel*, extracts treated with endoglycosidase Hf (Endo-Hf), which cleaves only high mannose *N*-linked polysaccharides present on immature H chains in the ER (*lower band of doublet*); mature H chains contain complex, *trans*-Golgi-derived *N*-linked polysaccharides that are insensitive and are responsible for its slower migration (*upper band of doublet*). *D*, Western blot of κ and λ1 L chains in the medium and in total cell lysates of transiently transfected HEK293 cells (*upper panels*) or infected v-abl pro-B cells (*lower panel*) with the indicated H chain and κ or λ1 L chain expression constructs. The VpreBΔC-JCλ5 fusion protein F1 cannot be detected with the anti-λ1 Ab; however, it was detected in the total lysate but not in the medium with rat anti-mouse Ab against λ5 (data not shown).

the relative amounts of mature, *trans*-Golgi-modified μ H chains (Fig. 3*C*), indicating this was at the level of ER export. These differences between L chains were also evident with the human μ H chain TG.SA, which can form BCRs with mouse κ and λ L chains that support B cell development in mice (Fig. 3, *A* and *B*, referred to as "T") (16, 17, 32). As in HEK293 cells, Dμ-F4^{SEC} and Dμ-λ1 receptors were detected on the surface (Fig. 3*B*; λ1 stains), and no surface Dμ-F2^{NS} complexes could be detected (Fig. 3, *A* and *B*). This corresponded to the appearance of the *trans*-Golgi-modified Dμ species only in Dμ-F4- and Dμ-λ1-expressing cells (Fig. 3, *C* and *D*). However, relative surface levels of these Dμ receptors were much less than the corresponding mouse and human μ receptors (Fig. 3, *A* and *B*), being barely detectable with the anti-IgM Abs, and despite being expressed comparably to μ receptors in HEK293 cells. These results imply that there are differences in the folding, assembly, and transport of H chains and L chains within the secretory pathway of these two cell types; pro-B cells were more restrictive such that receptor biosynthesis was more sensitive to L chain-secretion competency and H chain structure.

Intrinsic incompatibility of κ sequences with Dμ prohibits Dμ-κ L chain surface receptor formation

Previous studies from our laboratory and those of others have shown that Dμ cannot productively associate with two representative κ L chains (15, 16). The full-length κ L chains used in those studies were secretion incompetent, implying that they required a H chain partner to fold properly. Following the λ1 paradigm, it would have been predicted that on this basis those κ L chains would not form a receptor with Dμ. However, Dμ was not able to form a receptor complex with JCκ, the truncated, V_L-less counterpart of JCλ5 (17). However, it was unclear whether JCκ could not associate with Dμ because it could not fold and be secreted like JCλ5, or because there were incompatibilities with κ sequences.

Therefore, the truncated JCκ and a panel of κ L chains were tested for their secretion competency and ability to form surface Dμ-κ receptors. The secretion-incompetent κ1:MOPC21κ we used previously (16) contained a His residue within the V region that is a Tyr or Phe in most κ L chains. Substitution of this His

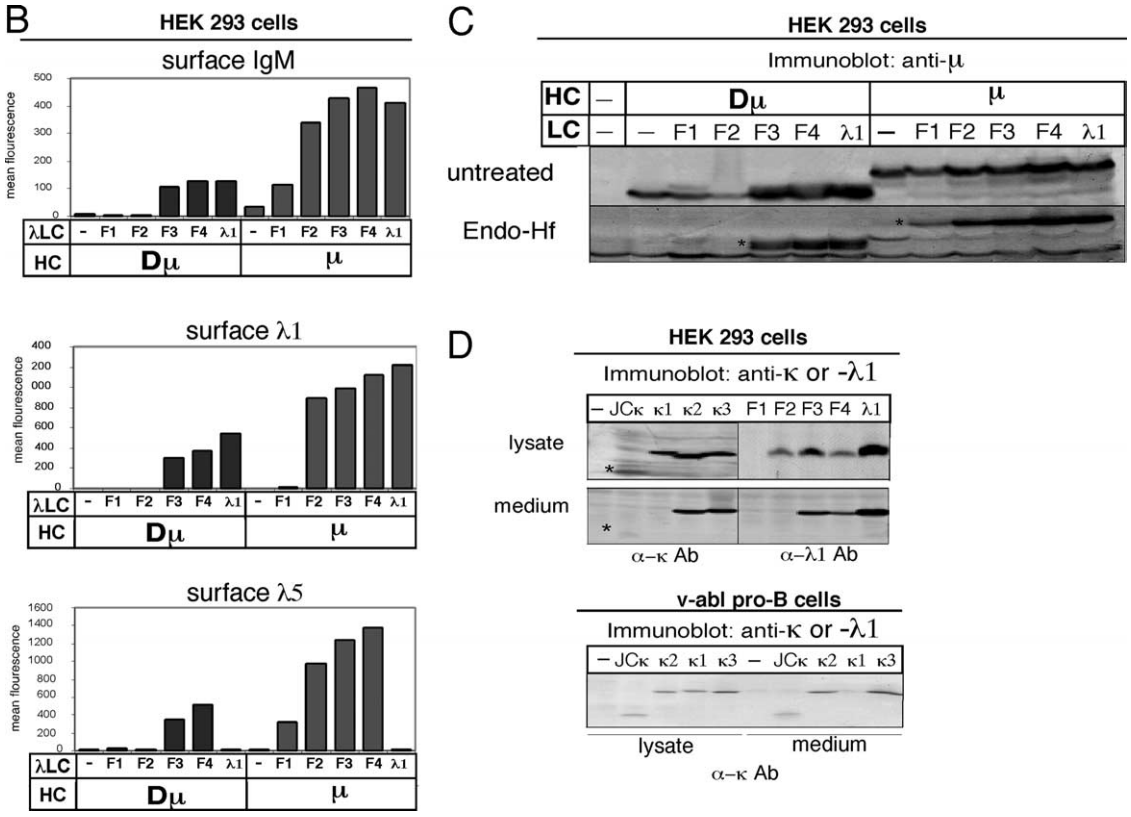

FIGURE 2. (*continued*)

residue in the V region of a nonsecreted VJκ-Cλ1 fusion protein into Phe or Tyr converted this fusion L chain from a nonsecreted into a secreted form (33). Based on this, the His in κ1 was converted to Tyr to create κ3:secκ. Also tested was the V region from a secreted κ L chain from a MPC11 myeloma cell line (κ2:MPC11κ; Fig. 1) (25).

As shown in Fig. 2*D*, JCκ, κ2:MPC11κ, and κ3:secκ, but not κ1:MOPC21κ, were detected in the medium of transfected HEK293 and infected v-abl transformed pro-B cells, even though all were expressed at comparable levels intracellularly. In primary pro-B cells, these κ L chains all formed complexes with the mouse μ H chain that were expressed on the surface, with μ-κ2:secκ and μ-κ3:MPC11κ BCRs at higher surface μ levels than μ-κ1:MOPC21κ or μ-JCκ (Fig. 3*A*; the μ-JCκ complexes contain endogenous VpreB, while the μ-λ complexes do not; see Ref. 17). Western blot analysis indicated this directly correlated with differences in the relative amounts of mature, endo-H-resistant μ proteins (Fig. 3*D*). The human μ H chain TG.SA (T) also could form surface receptors with the full-length κ L chains, although it was not able to form a surface receptor complex with JCκ, as was shown previously (Fig. 3*A*) (17). There was no detectable surface H chain detected by IgM staining on the surface of cells expressing Dμ with any of the κ L chains irrespective of their secretion competency. Correspondingly, only the immature form of Dμ was detected by Western blot in all cases (Fig. 3*D*). These results support the idea that κ L chains are categorically incapable of productively associating with Dμ to form surface receptors.

Dμ-L chain and μ-L chain receptor activity generally but not absolutely correlates with relative levels of ER export and surface expression

The signaling competency of μ and Dμ complexes was evaluated by how well they promoted preBCR or BCR-dependent growth and differentiation of primary Rag1$^{-/-}$λ5$^{-/-}$ pro-B cells. In these cells, de novo receptor expression via retroviral transduction of missing receptor components induces CD2 and CD22 expression and promotes proliferation and survival (17). Among the mouse and human μ-λ BCRs, the μ-λ1 and μ-F4^{SEC} receptors were the most active, displaying similar levels of activity for each H chain, whereas μ-F2^{NS} receptors were less active (Fig. 4*A–C*). This corresponded to the lower levels of mature and surface-expressed μ-F2^{NS} complexes in pro-B cells compared with the other μ-λ BCRs (Fig. 3*A–C*). However, whereas μ-λ1 and Dμ-λ1 complexes exhibited comparable levels of activity in the proliferation assay, Dμ-F4^{SEC} complexes were less active than Dμ-λ1, and differences between μ and Dμ complexes in activating CD2 and CD22 expression were more pronounced with F4^{SEC} than with λ1 (Fig. 4*A–C*). These differences paralleled the lower surface and maturation levels of Dμ with F4^{SEC} compared with λ1 (Fig. 3*A–C*). Cells expressing Dμ-F2^{NS} did not induce CD2 or CD22 or outgrow Rag1$^{-/-}$λ5$^{-/-}$ pro-B cells, being indistinguishable from Rag$^{-/-}$λ5$^{-/-}$ pro-B cells infected with GFP-only, H chain, or L chain viruses alone, and consistent with no ER export or surface expression of this complex.

Overall, μ-κ BCR complexes were also active for signaling in a manner that paralleled relative post-ER H chain maturation and surface receptor expression levels, with μ-κ3 complexes being the most active and on par with μ-λ1 in all cases (Fig. 4*D–F*). One difference between the mouse μ and human μ H chains was with JCκ, which only productively associated with the mouse H chain. Similarly, no signaling activity above BCR$^-$ controls was detected in cells coexpressing any of the κ L chains in conjunction with Dμ (Fig. 4*D–F*), consistent with the observation that none of the κ L chains was able to promote ER export of Dμ (Fig. 3*D*).

Interestingly, differences in surface expression and H chain maturation did not always account for some observed differences in

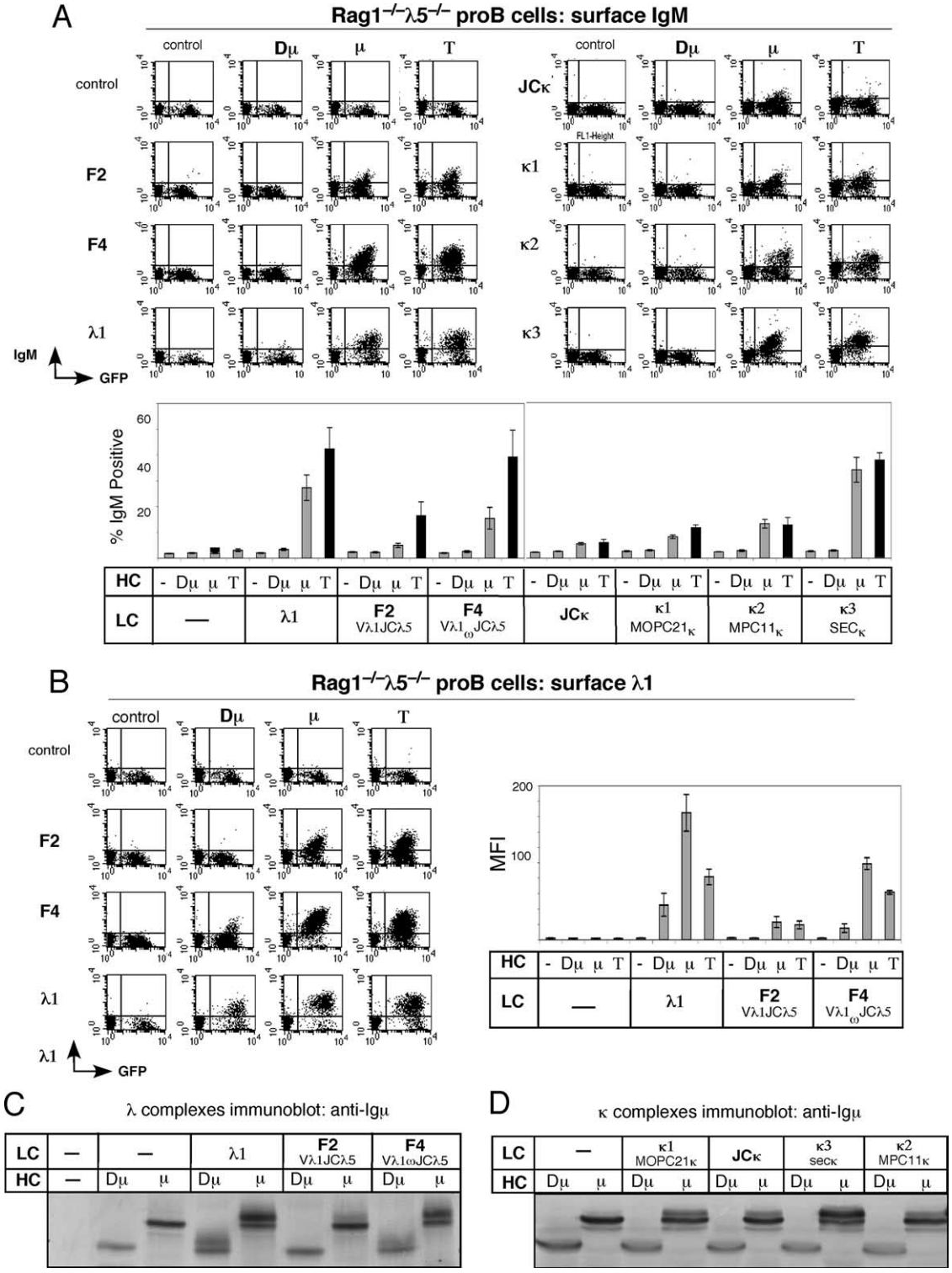

FIGURE 3. λ1 L chain's autonomous folding ability correlates with an increase of surface BCR levels in association with full-length and Dμ H chains, whereas κ L chains can associate only with the full-length H chains in primary pro-B cells. *A* and *B*, Flow cytometry analyses of surface BCR expression by Rag1$^{-/-}$λ5$^{-/-}$ pro-B cells expressing the indicated H chain and/or L chain via retroviruses, as detected either with Abs against IgM (*A*) or λ1 (*B*). Shown are representative panels of dot plots, with GFP expression plotted on the *x*-axis marking infected cells, and bar graphs plotting the average relative IgM or λ1 surface staining of GFP$^+$ cells for each sample set ($n \geq 3$, with SE bars shown). In *A*, IgM stain values of human TG.SA (T) are shown in black in the bar graph to indicate that the human and mouse IgM stains cannot be directly compared with each other because different detecting Abs were required for each. *C* and *D*, Immunoblots of mouse μ and Dμ H chain protein expression in primary Rag1$^{-/-}$λ5$^{-/-}$ cells double-infected with the indicated H chain-expressing retrovirus plus (*C*) control or the indicated λ L chain or (*D*) control or the indicated κ L chain.

receptor activity. Specifically, μ-κ1 and μ-κ2 complexes were expressed at similar or greater levels on the surface than was μ-F2^{NS} (Fig. 3*A*, IgM stains). They induced CD2 and CD22 expression as well as μ-F2^{NS}, but they were less active than μ-F2^{NS} for proliferation (Fig. 4). Additionally, whereas the mouse μ H chain showed about the same activity in association with κ1 and κ2 (Fig.

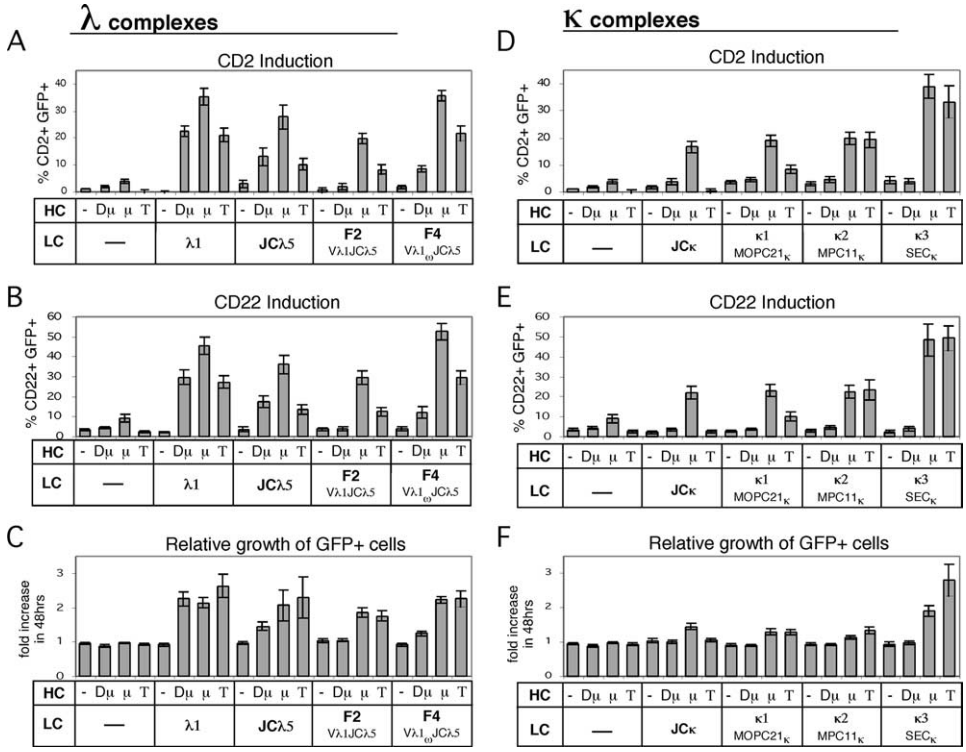

FIGURE 4. Activity of BCR receptor complexes in primary pro-B cells. Rag1$^{-/-}$λ5$^{-/-}$ pro-B cells were double-infected with retroviruses expressing the indicated H chains alone or plus λ (*A–C*) or κ (*D–F*) L chains, and infected cells (GFP$^+$) were evaluated after the appropriate culture period for CD2 expression (48 h later, *A* and *D*), CD22 expression (24 h later, *B* and *E*), and relative fold increase in population compared with GFP$^-$ cells (48 h later, *C* and *F*). The H and L chains expressed in each sample are indicated below each bar ($n \geq 6$ for all except κ3:secκ, for which $n = 3$; SE bars are shown).

4*D–F*), the human μ H chain T-κ1 receptor was less able to induce CD2 and CD22 expression than T-κ2, even though surface expression levels of the two complexes were equivalent (Fig. 3*A*, filled bars). Therefore, the clonotypic structure of the Ig components may also influence BCR activity.

Dμ-λ1 is less able to synergize with the IL-7R than the mouse μ-λ1 at low concentrations of IL-7

The above and previous findings indicated that Dμ-λ1 and the mouse and human μ-λ1 complexes exhibited comparable abilities to support Rag1$^{-/-}$λ5$^{-/-}$ B cell growth, which was surprising considering that mature B cells coexpressing exclusively Dμ and λ1 were not reported in mice when Dμ was expressed from a transgene (13, 24). The experiments found in Ref. 17 and in Fig. 4 were performed in the presence of 100 U/ml IL-7, a high concentration that supports robust pro-B and pre-B cell growth. At lower amounts (0.1 U/ml range) there is significant cell loss in both preBCR$^+$ and preBCR$^-$ cells, but pre-B cell growth and survival are more strongly favored due to synergy between the preBCR and IL-7R signaling pathways (29, 30, 34), conditions thought to better represent the physiological environment. Under these more stringent conditions, the Dμ-λ1 receptor was less active than the mouse μ and more comparable to the human, a profile resembling their relative activities in inducing CD2 and CD22 expression.

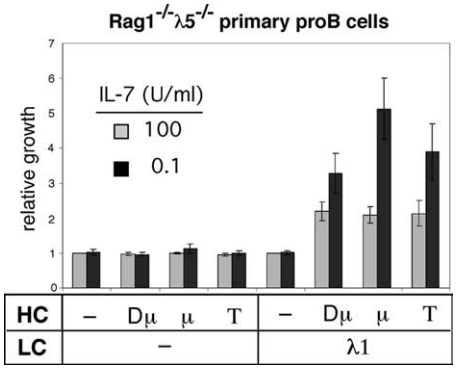

FIGURE 5. Dμ-λ1 and TG.SA-λ1 complexes are less active than μ-λ1 at promoting survival at low IL-7 concentrations. Relative growth of GFP$^+$CD19$^+$ primary Rag1$^{-/-}$λ5$^{-/-}$ pro-B cells double-infected with H chain and/or λ1 retroviruses at high (100 U/ml) and low (0.1 U/ml) IL-7 concentrations. Relative fold increase in GFP$^+$ cell number in 4 days was calculated as indicated in *Materials and Methods*. $n = 5$, SE bars are shown.

Discussion

Dμ provides a unique example of how Ig H chain and surrogate and conventional L chain structure influence receptor signaling and selection of the adult Ig H chain repertoire. At the pre-B cell stage, the Dμ-preBCR signaling impairment appears to primarily affect expression of maturation markers and proliferation whereas allelic exclusion is relatively intact. The findings in this study now suggest that even if Dμ-preBCRs up-regulate L chain germline transcription and rearrangement (12, 13), Dμ appears to be structurally incompatible with any κ and secretion-incompetent λ L chains. Moreover, the data also imply that not only is the Dμ H chain impaired to utilize a broad spectrum of L chains, but even if compatible λ L chains were made, the resulting Dμ-λ receptors would be less able to support development and growth than μ-λ receptors. Thus, multiple mechanisms appear to impede the emergence of Dμ alleles in the mature B cell repertoire.

Interestingly, the mechanisms restricting Dμ-BCR formation were different for the κ and λ L chains. The restrictive entity in λ chains was the V_L region, which had to maintain secretion competence to allow receptor formation with Dμ. In contrast, secretion competency was irrelevant for the κ chains, and the inability to form Dμ-κ complexes was due to general incompatibility between κ and Dμ. By comparison, secretion competence of full-length κ L chains enhanced their ability to form BCRs with both mouse and human full-length H chains, particularly in primary pro-B cells.

Moreover, JCκ only formed receptors with the mouse but not human full-length H chain and not with Dμ, despite being secretion competent, whereas JCλ5 and JCλ1 were able to do so with all H chains (this study and data not shown). It therefore appears that the λ JC_L sequence endows the λ1 L chain with the ability to be more accommodating of H chain structure than κ L chains. This is consistent with the greater flexibility of λ vs κ L chains at "elbows" at the J-C junctional sequences (35). λ L chain usage is a characteristic frequently associated with edited B cells (36). We speculate that this property may reflect the imperative of B cells undergoing editing to self-rescue by L chain replacement with L chains that have the best chance of pairing with whatever H chain is present when the κ locus is exhausted.

Our studies also support the model that the structure of Ig molecules can affect the activity of surface receptors, because observed differences in surface expression could not always account for differences in BCR activity. For example, the ability of μ-κ1:MOPC21κ and μ-κ2:MPC11κ complexes to promote proliferation was less than μ-F2:Vλ1J$C\lambda$5 even though they were all expressed at similar surface levels (Fig. 3*A*). Similarly, although the surface expression profiles of mouse and human μ BCR complexes with different full-length L chains were nearly indistinguishable, the human BCRs were not always as active as the corresponding mouse BCRs, with activity frequently more comparable to Dμ-BCRs. We speculate that the impaired signaling properties of Dμ complexes may therefore be due to the combined actions of impaired release of Dμ complexes from the ER and manifestations of structural defects of surface Dμ-λ complexes. Indeed, this has been shown for λ1 BCRs from the SLJ strain of mice, which contain an amino acid polymorphism in the λ1 C_L region that renders the surface λ1 BCR complexes signaling impaired (37).

Although the data support the idea that Dμ-λ1 complexes would not promote adult mature B cell survival or differentiation as well as normal H chains, they were not inert nor did they cause deletion in our tissue culture models. This partial activity may therefore explain why H chain alleles using D_H RF2 are underrepresented rather than completely absent. Why then might exclusively Dμ-expressing B cells not be found when Dμ expression is enforced (24)? In addition to structural flaws in Dμ that impair signaling, λ1 BCRs in general may be more restricted in their ability to support B cell homeostasis compared with κ, as there is age-dependent loss of λ1-expressing B cells in λ1-transgenic mice via selection of cells that have silenced the transgene and expressed endogenous κ L chains (38). In Dμ-transgenic Rag^+ B cells, Dμ and endogenous μ H chains were coexpressed, but the Dμ protein remained in an immature form, consistent with it not pairing with a compatible L chain like λ1 (13, 24). Nevertheless, if Dμ-λ1 receptors are formed, we speculate that their signaling properties may only be compatible with particular B cell populations. One example is fetal B cell progenitors, in which Dμ-λ1 complexes may be a force for positive selection (20); another may be marginal zone B cells, which remained intact in Dμ-transgenic mice (24). Our tissue culture system can show whether any given clonotypic preBCR or BCR forms an active signal transduction complex. However, its readouts for receptor activity represent only a subset of changes characteristic of the pro- to pre-B cell transition. The in vitro system thus provides an important starting point for comparison to in vivo systems in which more elaborate and physiological execution of programs of B cell differentiation, proliferation, survival, allelic exclusion, and activation of the underlying signal transduction pathways can be used as parameters to compare the activity of receptors like Dμ-λ1 and μ-λ1.

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Disclosures

The authors have no financial conflicts of interest.

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