Brief Definitive Report

Surrogate or Conventional Light Chains Are Required for Membrane Immunoglobulin Mu to Activate the Precursor B Cell Transition

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Summary

To examine the role of light chains in early B cell development we combined RAG-1 and λ5 mutations to produce mice that expressed neither conventional nor surrogate light chains (RAG-$1^{-/-}$, $\lambda5^{-/-}$). Unique heavy and light chain genes were then introduced into the double and single mutant backgrounds. Membrane immunoglobulin (Ig)μ (mIgμ) associated with Igα-Igβ but was unable to activate the pre–B cell transition in RAG-$1^{-/-}$ $\lambda5^{-/-}$ mice. Either λ5 or kappa light chains were sufficient to complement this deficiency. Therefore light chains are absolutely required for a functional Ig signaling module in early B cell development. Our data provide direct evidence for the existence of two pathways for induction of early B cell development: one which is activated through surrogate light chains and mIgμ, and an alternative pathway which uses conventional light chains and mIgμ.

Lymphocyte development in the bone marrow advances through a series of ordered events, ultimately resulting in B cells that express Ig Ag receptors (1, 2). Although little is known about the initial lineage commitment events, progression along later stages in this pathway is guided by the expression of productively rearranged Ig heavy chains, and the membrane Igμ (mIgμ)-associated Igα and Igβ signal transducers (3, 4).

The earliest event thought to be induced by mIgμ is allelic exclusion (5). Upon a productive $V_H \rightarrow DJ_H$ rearrangement and subsequent mIgμ expression, further rearrangements at other heavy chain loci are inhibited, resulting in B cells with clonotypic receptors (6–8). Recent work in several laboratories has shown that in addition to mIgμ, Igα, and Igβ, the surrogate light chain λ5 is also required to establish allelic exclusion (9–11). Thus, allelic exclusion is thought to be regulated by a complex formed by mIgμ, λ5, V pre-B, and the Igα-Igβ signal transducer.

After activation of allelic exclusion, mIgμ induces progenitor B (pro-B) cells to progress to the next stage in development and become precursor B cells (pre-B) (6, 12, 13). The pre-B cell transition appears to be activated by the same B cell receptor (BCR) components that trigger allelic exclusion. Mice that cannot produce a functional mIgμ, (8, 14–16), or the Igα-Igβ signaling complex (3, 17, 18) exhibit a profound block in B cell development at the pro–B cell stage. In contrast the requirement for the surrogate light chain in the pre–B cell transition is not absolute and λ5 deficiency results in an incomplete block in development: $\lambda5^{-/-}$ pro–B cells leak through to the pre–B cell stage and ultimately populate the mature B cell compartment (19, 20). To explain the leaky phenotype found in $\lambda5^{-/-}$ mice, Melchers, Rajewsky, and colleagues have proposed that there are two pathways for B cell development (19, 21). Under normal circumstances mIgμ would be paired with the surrogate light chains, λ5 and Vpre-B, to produce an active pre–B cell receptor. Alternatively, when the surrogate chains are not expressed, or when a light chain rearranges early, a salvage pathway would be activated. This secondary pathway would require that mIgμ pair with conventional light chains to produce the functional pre-B cell receptor complex.

To test this hypothesis we studied B cell development in mice restricted to expressing mIgμ alone, or in combination with either κ light chain, or surrogate light chains. Here we report direct evidence for the existence of two pathways that mediate early B cell development.

Materials and Methods

Constructs and Mice. The transgenic constructs used for the experiments reported here have been described elsewhere (22). The idiotype produced by the heavy and light chain combination is detected with the 54.1 monoclonal antibody (23). RAG-$1^{-/-}$ mice were a gift from Dr. David Baltimore (Massachusetts Institute of Technology, Cambridge, MA) and E. Spanopoulou (Mount Sinai Medical School, New York). $\lambda5^{-/-}$ mice were a gift from Dr. F. Melchers (The Basel Institute for Immunology, Basel, Switzerland) and provided by Dr. M.D. Cooper (University of Alabama at Birmingham, Birmingham, AL). All mice were bred and maintained under specific pathogen-free conditions.

Flow Cytometry. The following antibodies were used for surface staining of bone marrow B cells: PE-B220 (Pharmingen, San

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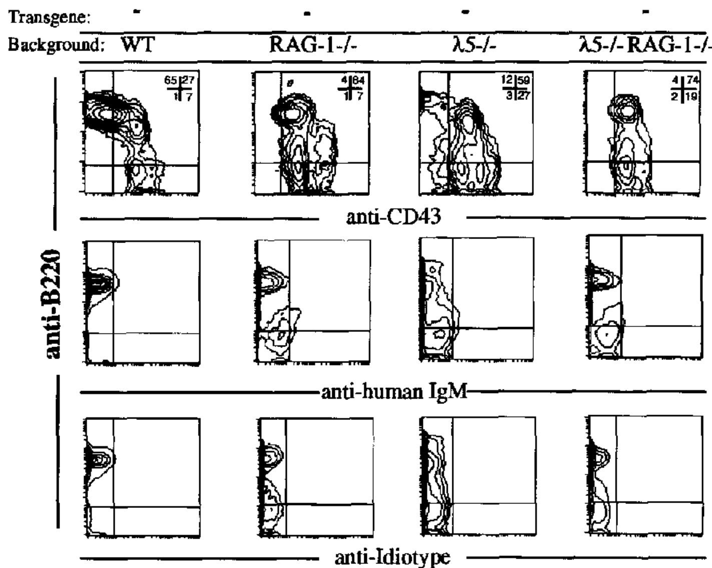
Diego, CA), FITC-CD43 (S7 hybridoma; American Type Culture Collection, Rockville, MD), biotin-labeled anti–human IgM (heavy chain–specific DA4.4 clone; Southern Biotechnology Associates, Inc., Birmingham, AL), and biotin-labeled antiidiotype (54.1 hybridoma; a gift from Dr. D.Nemazee, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Biotin-labeled antibodies were visualized with streptavidin-RED670 (GIBCO-BRL, Gaithersburg, MD). Data were collected on a FACScan® (Becton Dickinson and Co., Mountain View, CA) and were analyzed using CellQuest software (Becton Dickinson).

Intracellular Human IgM Expression. The presence of human IgM heavy chain in the cytoplasm of B cells was verified by FACS®, as previously described (24). Briefly, B cells from the bone marrow of 6–8-wk-old mice were first stained with PE-B220 and FITC-CD43 (S7 hybridoma) antibodies. The cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with 1% saponin. Anti–human IgM was then used to stain the treated B cells. Data acquisition was performed with a FACScan® and appropriate gating and analysis was done with CellQuest software.

Abelson Virus Transformation of Bone Marrow cells. Bone marrow from 6–8-wk-old mice was transformed with Abelson MuLV (A-MuLV) in accordance with published protocols (25). Briefly, 2×10^7 bone marrow cells were suspended in 6 ml of complete medium (RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 μM 2-ME), treated with 2 ml of virus stock, and plated onto 100-mm plastic petri dishes (Corning Inc., Corning NY). The dishes were left at 37°C in a humidified 5% CO_2 atmosphere for 2 h, and then 4 ml of medium was added. Transformed B cells were maintained in complete medium.

Immunoprecipitation and Immunoblotting. A-MuLV–transformed bone marrow cells were lysed in 1% *n*-dodecyl-B-D-maltoside



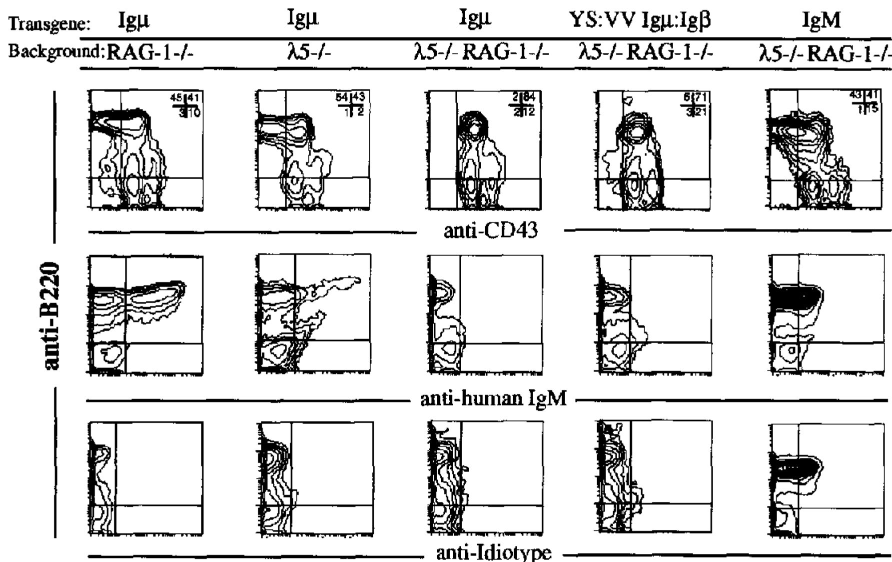


Figure 1. Bone marrow cells from 6–8-wk old mice were analyzed by staining with PE–anti-B220, FITC–anti-CD43, biotin-anti–human IgM and biotin-anti–idiotype (54.1) antibodies. The lymphocyte population was gated according to standard forward- and size-scatter values. For the PE–anti-B220/ FITC–anti-CD43 profiles, the numbers on the upper left corner represent the percentages of gated lymphocytes in each quadrant. (*A*) FACS® profiles from all the control animals. (*B*) B cell development in the bone marrow of transgenic mice deficient for RAG-1 and λ5.

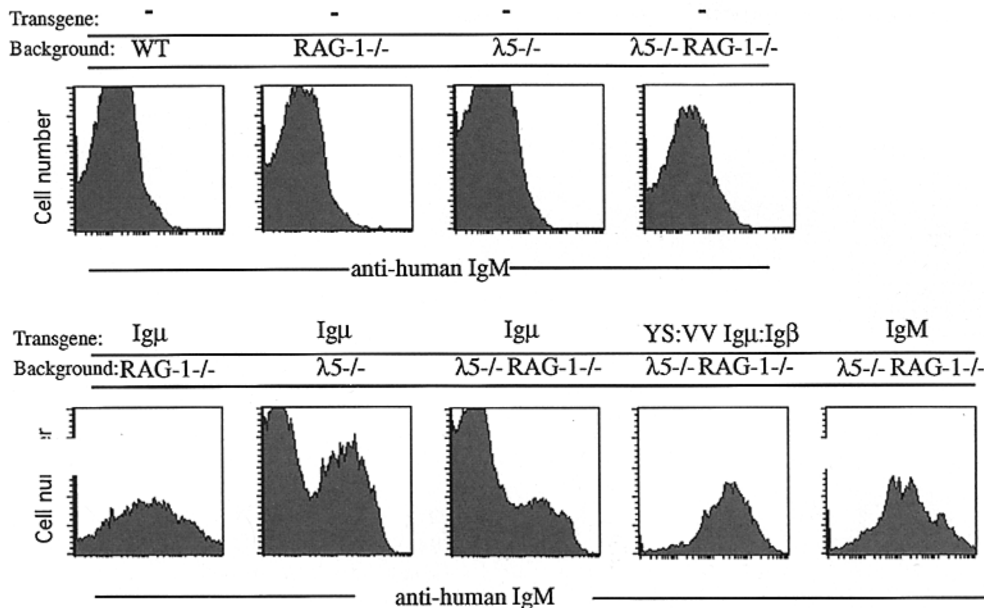


Figure 2. Intracellular expression of the human μ heavy chain transgene. Cells, stained with PE–anti-B220 and FITC–anti-CD43, were fixed, permeabilized, and subsequently stained with biotin-anti–human IgM. Gating was on $CD43^+$ cells

(Anatrace, Maumee, OH), 50 mM Tris, pH 6.8, 100 mM NaCl. Insoluble material was removed by centrifugation. The supernatant was incubated with rabbit antiserum to human IgM ($5F_c{}^m$) (Cappel Research Products, Durham, NC) and protein A agarose (Pierce Chemical Co., Rockford, IL) for 2 h at 4°C. Immune complexes were collected, washed in lysis buffer, separated by 10% reducing SDS-PAGE, and transferred to Immobilon-P (Millipore Corp., Bedford, MA) in a semi-dry system (Owl Scientific, Cambridge, MA). After incubation with anti–human IgM (Cappel), anti-Igα, or anti-Igβ (26) blotting antibodies, labeled proteins were visualized with ^{125}I-protein A (New England Nuclear, Boston, MA) using x-ray film.

Results

Light Chains Are Necessary for the Pro-B to Pre–B Cell Transition. In the bone marrow, induction of the pre–B cell transition coincides with loss of expression of CD43, and so pre–B cells are $B220^+CD43^-$ whereas pro–B cells are $B220^+CD43^+$ (Fig. 1 *A* and [27]). To determine whether light chains are required for early B cell development we first bred together RAG-$1^{-/-}$ and $\lambda 5^{-/-}$ targeted mice to create double-deficient animals (RAG-$1^{-/-}\lambda 5^{-/-}$). In this background there are no heavy or light chain gene rearrangements because of the RAG-1 deficiency (15), and λ5 is not expressed (19, 20). The phenotype of the RAG-$1^{-/-}\lambda 5^{-/-}$ animal resembles that of the RAG-$1^{-/-}$ mice, in that B cells are arrested at the $B220^+CD43^+$ pro–B cell stage (Fig. 1 *A*).

The developmental defect observed in the RAG-$1^{-/-}$ mice is strictly due to their inability to perform Ig rearrangements, and it can be overcome by the addition of a pre-rearranged mIgμ transgene (12, 13). The same human mIgμ transgene also facilitated the pre–B cell transition in $\lambda 5^{-/-}$ mice (Fig. 1 *B*). However, it had no effect in the double-mutant background: FACS® analysis of the bone marrow of TG.mIgμ RAG-$1^{-/-}\lambda 5^{-/-}$ mice revealed only $B220^+CD43^+$ pro–B cells (Fig. 1 *B*). These cells expressed human Igμ but did not transport it to the cell surface: human mIgμ was entirely intracellular in TG.mIgμ RAG-$1^{-/-}\lambda 5^{-/-}$ pro–B cells (Fig. 2) as well as in TG.mIgμ RAG-$1^{-/-}\lambda 5^{-/-}$ A-MuLV–transformed B cell lines (data not shown). Since the only difference between the TG.mIgμ RAG-$1^{-/-}$ and the TG.mIgμ RAG-$1^{-/-}\lambda 5^{-/-}$ mice is the expression of λ5 in the former, we conclude that in the absence of conventional light chains the surrogate chains are absolutely required to deliver the signal for B cell development.

To determine whether κ light chains could substitute for surrogate light chains in the double-mutant mice we combined a κ light chain transgene with mIgμ transgene (mIgμ+κ) in the RAG-$1^{-/-}\lambda 5^{-/-}$ double-deficient mice. Addition of the light chain was found to rescue B cell development in this background: the bone marrow of TG.mIgμ+κ RAG-$1^{-/-}\lambda 5^{-/-}$ mice contained $B220^+$ $CD43^-$ pre–B cells, which carried transgenic IgM on their surface and stained positive for the antiidiotypic antibody 54.1 (Fig. 1 *B*). Therefore, the κ light chain is able to reconstitute B cell development by substituting for λ5 in RAG-$1^{-/-}\lambda 5^{-/-}$ double-deficient mice.

The Pre-BCR Is Assembled but Does Not Function in the Absence of Light Chains. One explanation for the observation that mIgμ is inactive in the RAG-$1^{-/-}\lambda 5^{-/-}$ background might be that light chains are required for assembly of the early BCR. To determine whether mIgμ was associated with Igα-Igβ in the absence of light chains we performed immunoprecipitation experiments on A-MuLV–transformed cells derived from TG.mIgμ RAG-$1^{-/-}\lambda 5^{-/-}$ bone marrow. We found no difference in the amount of Igα and Igβ that was associated with the heavy chain in the presence or absence of light chains (Fig. 3). However, it was only in the TG.mIgμ RAG-$1^{-/-}$-derived lines that we saw the fully glycosylated surface form of Igβ coprecipitate with human IgM (Fig. 3). The two glycosylated Igβ bands

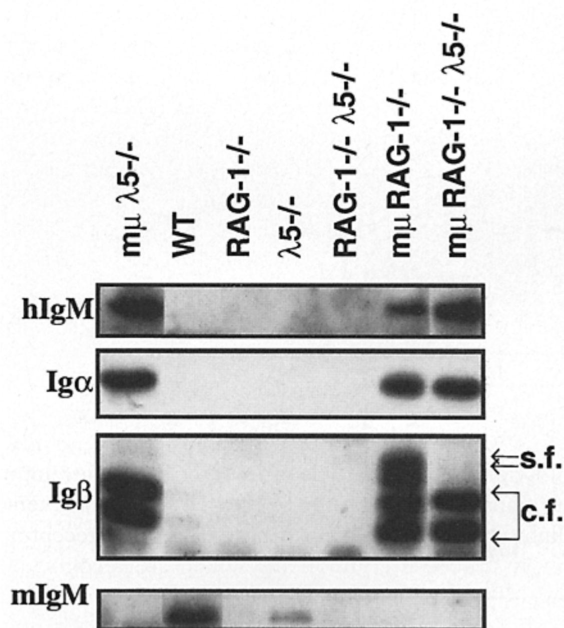


Figure 3. Light chains are not necessary for proper assembly of the early BCR, since Igα and Igβ can associate with mIgμ in their absence. Ig receptor complexes were immunoprecipitated from A-MuLV transformed B cells derived from the bone marrow of transgenic mice. Immunoprecipitations were done with protein A- coupled rabbit anti–human IgM and the membranes were subsequently blotted with anti–human IgM, anti–mouse IgM (control), anti-Igα and anti-Igβ antibodies (26). *s.f.*, surface form; *c.f.*, cytoplasmic form.

were absent in the lanes which represent precipitates from TG.mIgμ RAG-$1^{-/-}$λ$5^{-/-}$-derived B cell lines (Fig. 3), and only visible after prolonged exposure in TG.mIgμ λ$5^{-/-}$ A-MuLV cell lines which only rarely rearrange and express conventional light chains (not shown). All four Igβ bands collapse into one upon digestion with endoglycosidase H. Thus association of Igα-Igβ with the heavy chain is light chain independent, but cell surface transport requires either λ5 or conventional light chains.

To directly assess whether pre-BCR function requires light chains we introduced a mIgμ:Igβ chimeric transgene into the RAG-$1^{-/-}$λ$5^{-/-}$ background (TG.mIgμ:Igβ RAG-$1^{-/-}$λ$5^{-/-}$). The Igμ component of this transgene encodes a membrane-bound Ig with a transmembrane mutation that prevents its normal association with Igα-Igβ. The cytoplasmic domain of the chimeric protein is encoded by Igβ. Signaling by the chimeric mIgμ:Igβ transgene is through tyrosine residues in the Igβ tail and is independent of endogenous Igα-Igβ (22). Thus, this chimeric receptor is preassembled into a functional subunit and does not have to associate with Igα-Igβ to induce the pre–B cell transition. Although the chimeric receptor was active in the RAG-$1^{-/-}$ background (22) it was completely inactive in RAG-$1^{-/-}$λ$5^{-/-}$ mice (Fig. 1 *B*). There were no $B220^{+}CD43^{-}$ cells in the bone marrow of TG.mIgμ:Igβ RAG-$1^{-/-}$λ$5^{-/-}$ mice (Fig. 1 *B*). In addition transgenic mIgμ:Igβ was not expressed on the cell surface in the double-mutant background although transgenic mIgμ was found in large quantities in the cytoplasm of TG.mIgμ:Igβ RAG-$1^{-/-}$λ$5^{-/-}$ pro–B cells (Fig. 2). We conclude that light chains are not required to assemble a pre-BCR complex and must therefore be necessary for some other limiting step, such as cell surface transport or interaction with an as yet unknown cognate receptor.

Discussion

The antigen receptor found on mature B cells is a complex structure. It is composed of an Ig heavy and light chain heterodimer, and the Igα-Igβ signal transducers which are noncovalently associated with mIgμ (28). The heavy and light chains bind antigen but have no signaling capacity on their own, and require Igα and Igβ to induce the phosphorylation of non–receptor tyrosine kinases which give rise to physiologic responses (26, 29–33).

A receptor similar to the mature BCR is thought to mediate several key developmental events in early B cells. As in the mature BCR the signaling module of the pre-BCR is the Igα-Igβ heterodimer: both Igα and Igβ are expressed very early in B cell development (34–36), and they associate with mIgμ as soon as it is produced (this paper, and [28]). Furthermore, the Igα and Igβ proteins are necessary and sufficient to induce early B cell development (17, 18, 22, 24), and the mechanism by which the Igα-Igβ heterodimer activates early B cell transitions is analogous to that used by the mature BCR (31–33). Both have a requirement for receptor tyrosine phosphorylation (22, 24), syk kinase signaling (37, 38) and receptor cross-linking. However, the molecular identity of the pre-BCR ligand, as well as the receptor components required for recognition, have been the subject of debate. For example, results from gene-targeting experiments (19, 20) as well as experiments with transformed B cell lines (39) suggested that the surrogate light chains λ5 and VpreB are important components of the early B cell receptor. In addition, signaling by the early B cell receptor appears to be dependent on λ5 in pro–B cells, as there is no allelic exclusion in these cells in λ$5^{-/-}$ mice (9, 10). But despite the lack of allelic exclusion in pro–B cells in λ$5^{-/-}$ mice, the few B cells that do mature in this mutant background express a single heavy and light chain combination (19). One way to account for the differences in allelic exclusion between λ$5^{-/-}$ pro–B cells and λ$5^{-/-}$ mature B cells would be if the combination of mIgμ with light chains could also produce a functional pre–B cell receptor. According to this model the mIgμ-Igα-Igβ signaling module of the pre–B cell receptor would be recognized and triggered when combined with either a conventional or a surrogate light chain.

Consistent with this idea, targeting of the λ5 gene does not abrogate B cell development completely (19), and so λ5 is not indispensable for either allelic exclusion or B cell development. Our finding that a heavy chain transgene could facilitate the pre–B cell transition in λ$5^{-/-}$ mice is in agreement with Grawunder et al. (40) though not with Corcos et al. (11). Both our results and those reported by Grawunder (40) support the idea that the leaky phenotype in λ$5^{-/-}$ mice can be explained by stochastic V(D)J rear-

rangements. Producing in-frame VDJ_H and VJ_L combinations in any single developing B cell is a rare event, yet it would be essential for pre–B cell development to proceed in the absence of λ5. Thus it would be expected that introducing an already rearranged heavy chain into the $\lambda5^{-/-}$ background would increase the number of pre–B cells by reducing the requirement for activation of the transition to successful completion of light chain gene rearrangements. Our finding that a transgenic κ light chain was required for mIgμ to activate the pre–B cell transition in RAG-$1^{-/-}\lambda5^{-/-}$ mice provides further support for this idea, because it shows that in the absence of the surrogate light chains, conventional light chains are indeed essential for pre-BCR function in early B cell development. When neither surrogate light chains nor conventional light chains are expressed, B cell development cannot proceed past the pro–B cell stage.

What is the role of the light chain in developing B cells? Our experiments show that light chains are not simply facilitators for assembly of the mIgμ, Igα-Igβ complex. The mIgμ transgene that was inactive in RAG-$1^{-/-}\lambda5^{-/-}$ pro–B cells, was associated with Igα-Igβ. Further, a chimeric mIgμ:Igβ transgene, that encodes a preassembled BCR that does not require endogenous Igα-Igβ for signaling, was also inactive in the RAG-$1^{-/-}\lambda5^{-/-}$ mice. Therefore, it appears that λ5 or conventional light chains are not necessary for pre–B cell receptor assembly but they are required to produce a receptor which is competent to signal.

Studies with secreted Igs and B cell lines have shown that light chains are required to release the heavy chain from immunoglobulin binding protein in the endoplasmic reticulum (41) and for heavy chain transport (42–44). Mutant cell lines that do not produce light chains fail to secrete the heavy chain (42–44). This transport function appears to be independent of assembly and antigen binding since point mutations in conserved light chain residues alter transport but not antigen binding (45). If signaling by the early BCR is a cell surface event, then the role of the light chain, either surrogate or conventional, may be limited to transporting the BCR complex to the cell surface. The observation that the inactive mIgμ–Igα-Igβ complex remained intracellular in both bone marrow and A-MuLV–transformed RAG-$1^{-/-}\lambda5^{-/-}$ B cells supports the hypothesis that one of the functions of the light chain in early B cell development is to transport the receptor to the cell surface. Nevertheless, light chains may also contribute directly to receptor activation as structural components which are recognized by the putative cross-linker either on the cell surface or inside the developing B cell. In this model the cross-linker must see a structural element shared by all conventional light chains as well as surrogate light chains. The availability of RAG-$1^{-/-}\lambda5^{-/-}$ pro–B cells which can be complemented with combinations of altered Ig transgenes should facilitate the elucidation of the molecular mechanisms that control early BCR function.

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