International Immunology, Vol. 14, No. 6, pp. 577–584

The variable, C_H1, C_H2 and C_H3 domains of Ig heavy chain are dispensable for pre-BCR function in transgenic mice

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Keywords: B cell development, pre-B cell receptor, allelic exclusion, V(D)J recombination, transgenic mouse

Abstract

The pre-BCR consists of Ig μ protein, the product of a heavy chain gene assembled by V(D)J recombination in pro-B cells, the surrogate light chains $V_{pre\text{-}B}$ and λ5, and the signaling chains Igα and Igβ. Signaling by the pre-BCR is a checkpoint required for further maturation of pro-B cells in the adult bone marrow. However, it is currently not known whether an extracellular ligand is required to initiate pre-BCR signaling. We reasoned that if the ectodomain of the pre-BCR is required to interact with a ligand, then a truncated heavy chain protein would not support B cell development. To test this notion, we produced transgenic mice expressing a heavy chain protein whose extracellular domains except for C_H4 were replaced by an irrelevant Ig superfamily ectodomain from the human CD8α protein. This transgene resulted in pre-BCR-like signaling since it rescued development of pre-B cells in recombinase-activating gene (RAG)1-deficient mice and resulted in allelic exclusion of the endogenous Ig heavy chain gene in RAG-proficient mice. These findings lead us to suggest that the majority of the extracellular region of the pre-BCR is not required for pre-BCR function and, thus, ligand binding is unlikely to be required for pre-BCR function.

Introduction

Lymphoid development is characterized by the highly regulated assembly of antigen receptor genes from their component gene segments via a process known as V(D)J recombination. In developing B cells, the Ig heavy chain (IgH) locus rearranges before the Ig light chain loci, with D-to-J_H rearrangement preceding V-to-DJ_H rearrangement. In developing T cells, the TCRβ locus rearranges before the TCRα locus, with D-to-$J_β$ rearrangement preceding V-to-$DJ_β$ rearrangement. If IgH or TCRβ chain rearrangement results in an 'in-frame' allele, Ig light chain or TCRα chain rearrangement is activated and further IgH or TCRβ chain rearrangement ceases. This latter observation contributes to a molecular explanation of the well-known phenomenon of allelic exclusion—an individual B or T cell expresses only one functional antigen receptor.

Expression of IgH or TCRβ chain protein marks a critical checkpoint in B and T cell development. Previous work has shown that these chains assemble into a plasma membrane-associated structure known as the pre-BCR or pre-TCR consisting of surrogate light chains ($V_{pre\text{-}B}$ and λ5) or surrogate α chain (pre-Tα) and the accessory chains Igα, Igβ or the CD3 complex respectively [reviewed in (1–3)]. Mutation in any component of the pre-antigen receptor complexes results in a block in progression of development in that lineage. Therefore, understanding how the pre-BCR and pre-TCR transmit their signals is critical for understanding ordered gene rearrangement and allelic exclusion.

It is possible that a ligand exists in the developing cell environment which activates pre-BCR or pre-TCR signaling. Alternatively, signaling may be triggered by plasma membrane assembly of the receptor complex itself without need for an exogenous ligand. There is strong support for this latter notion with respect to the pre-TCR. Developing thymocytes expressing truncated mutant TCRβ and pre-Tα chains containing only affinity tags in place of their ectodomains nonetheless support apparently normal T cell development (4). The situation in developing B cells is less clear. Our group and others have shown that a truncated heavy chain transgene, incapable of binding surrogate light chains, can nonetheless promote the pro-B to pre-B transition (5,6). It remained

Corresponding editor: M. S. Schlissel; E-mail: mss@uclimk4.berkley.edu

Transmitting editor: M. Bevan

Received 10 December 2001, *accepted* 28 February 2002

possible, however, that a putative pre-BCR ligand could interact with the C_H2, C_H3 or C_H4 domains of heavy chain protein remaining in those truncated heavy chain transgenic proteins. In order to clarify the potential role of a ligand in pre-BCR signaling, we have analyzed a new transgenic mouse line which expresses a chimeric heavy chain transgene encoding a protein consisting of the human CD8α (huCD8α) ectodomain fused to the membrane proximal portion of C_H4 and the μ chain transmembrane exons. We find that in a fashion similar to the truncated pre-TCR noted above, the huCD8α–μ transgene promotes the pro-B to pre-B transition, indicating that pre-BCR signaling is independent of extracellular ligand.

Methods

Construction of plasmids

An *Eco*RI–*Xho*I restriction fragment of pHCMV-CD8/μ4m (7), a gift from A. Venkitaraman (CIMR, Cambridge, UK), encoding the huCD8–μ chimera was blunted with the Klenow fragment of DNA polymerase I and cloned into a blunted *Acc*I site in the $E_\mu V_H$ expression vector (8). The resulting $E_\mu V_H$–huCD8–μ expression cassette was then flanked by tandem pairs of the chicken β-globin insulator by cloning it (in place of the γ-neo cassette) into the *Bam*HI site of pJC13-1 (9), a gift of A. Bell and G. Felsenfeld (NIH, Bethesda,, MD). Prior to insertion of $E_\mu V_H$–huCD8–μ, pJC13-1 was first modified by removing the 5′ HS2 mouse globin LCR from the *Eco*RI site, blunting the *Eco*RI site using Klenow DNA polymerase and replacing it with a *Sal*I linker (NEB, Beverly, MA).

Production and maintenance of transgenic mice

A 13 kb *Sal*I–*Sal*I fragment containing the doubly insulated $E_\mu V_H$–huCD8–μ expression cassette was microinjected into the pronuclei of C57/BL6 × C3H zygotes (Johns Hopkins University School of Medicine Transgenic Core Facility, Baltimore, MD). Founder huCD8–μ transgenic mice were identified by PCR and Southern analysis of genomic DNA from tail. Founder huCD8–μ transgenic mice were bred to recombinase-activating gene (RAG)1-deficient mice (10) and in some cases to β2-microglobulin (β_2m)-deficient mice (Jackson Laboratories, Bar Harbor, ME). The mice were housed in microisolator cages according to NIH guidelines.

Genotyping

PCR assays were used to determine the genotype of each mouse as previously described (6). Below are sequences of PCR primers not previously described: huCD8–μ transgene (T = 62°C): HCD8L, 5′-GAC AGT GGA GCT GAA GTG CC; HCD8R, 5′-GGC TGC GAC GCG ATG GTG; β_2m knockout (T = 56°C): β_2mL, 5′-ACT CAC GCC ACC CAC CGG AG; β_2mR, 5′-GAT GCT GAT CAC ATG TCT CG. PCR cycling conditions were 94°C for 1 min; T annealing (as noted for each primer set above in parentheses) for 1 min; 72°C for 2 min, for 30 cycles.

Flow cytometric analysis of primary cells

Single-cell suspensions of bone marrow or spleen were stained for flow cytometry using the following antibodies (purified from hybridoma supernatant in this laboratory or purchased from PharMingen, San Diego, CA, Sigma, St Louis, MO or Ortho-Diagnostics, Raritan, NJ): CD19 (1D3), B220 (RA3-6B2), μ (331.2), CD43 (S7), huCD8α (UCHT-4 or OKT-8). The antibodies were either conjugated to FITC, phycoerythrin or biotin. Biotinylated antibodies were revealed using streptavidin–Quantum Red (Sigma). Antibody to mouse MHC I (II/41) was a gift from M. Soloski (Johns Hopkins). Stained cells were analyzed with a Becton Dickinson (Mountain View, CA) FACScan and CellQuest software. Dead cells were excluded by gating on live lymphocytes using forward and side scatter.

PCR analysis of V(D)J gene rearrangement and double-strand DNA breaks

Assays were performed as previously described (6).

Results

Expression of a chimeric huCD8–μ transgene in developing B cells

Previous studies had shown that a chimeric protein consisting of the entire ectodomain of hCD8α and the C_H4 and transmembrane domains of the Ig μ heavy chain is expressed on the surface of transfected B cell lines in association with the signaling molecules Igα and Igβ (7). In order to determine the role of the V_H and C_H1–3 domains of Ig heavy chain protein in B cell development, we generated transgenic mice using this huCD8–μ cDNA and the B cell-specific expression vector $E_\mu V_H$ (8). The presumed structure of a huCD8–μ-containing pre-BCR is shown in Fig. 1 in comparison with the wild-type pre-BCR and a previously studied truncated pre-BCR (6).

Founders positive for the transgene were bred to RAG1-deficient mice through two generations, and bone marrow and spleen purified from various individual pups were analyzed for transgene expression using an anti-huCD8 antibody and flow cytometry (Fig. 2). We found that a significant fraction of B lineage ($CD19^+$) bone marrow cells (~50%) and a small fraction of splenic B cells ($B220^+$; 2.6–18%) in both a $RAG1^{-/-}$ and $RAG1^{+/-}$ background expressed the transgene.

huCD8–μ expression facilitates differentiation of pro-B cells to pre-B cells

The pro-B to pre-B cell transition in B cell development is characterized by changes in cell surface expression of CD43 and CD2. $RAG1^{-/-}$ B cell progenitors are arrested at the $CD43^+$ $CD2^-$ stage of development (11–14). Expression of a full-length wild-type μ transgene in $RAG1^{-/-}$ mice causes these cells to progress to the pre-B cell stage ($CD43^-CD2^+$) (10,15). Similarly, we found that expression of huCD8–μ resulted in the loss of CD43 expression and the acquisition of CD2 expression (Fig. 3). In order to assess the efficiency of huCD8–μ in promoting the pro- to pre-B cell transition in $RAG^{-/-}$ mice, we compared the number of pro-B ($CD19^+CD43^+$) and pre-B ($CD19^+CD43^-$) cells in groups of $RAG^{-/-}$, $RAG^{-/-}$ huCD8–μ and $RAG^{-/-}$ wild-type–μ transgenic mice (Table 1). This analysis revealed that the mutant and wild-type–μ transgenes were quantitatively similar in their ability to promote the pro-to-pre B transition. These observations lead us to conclude that the pre-BCR signal resulting in alterations in surface marker

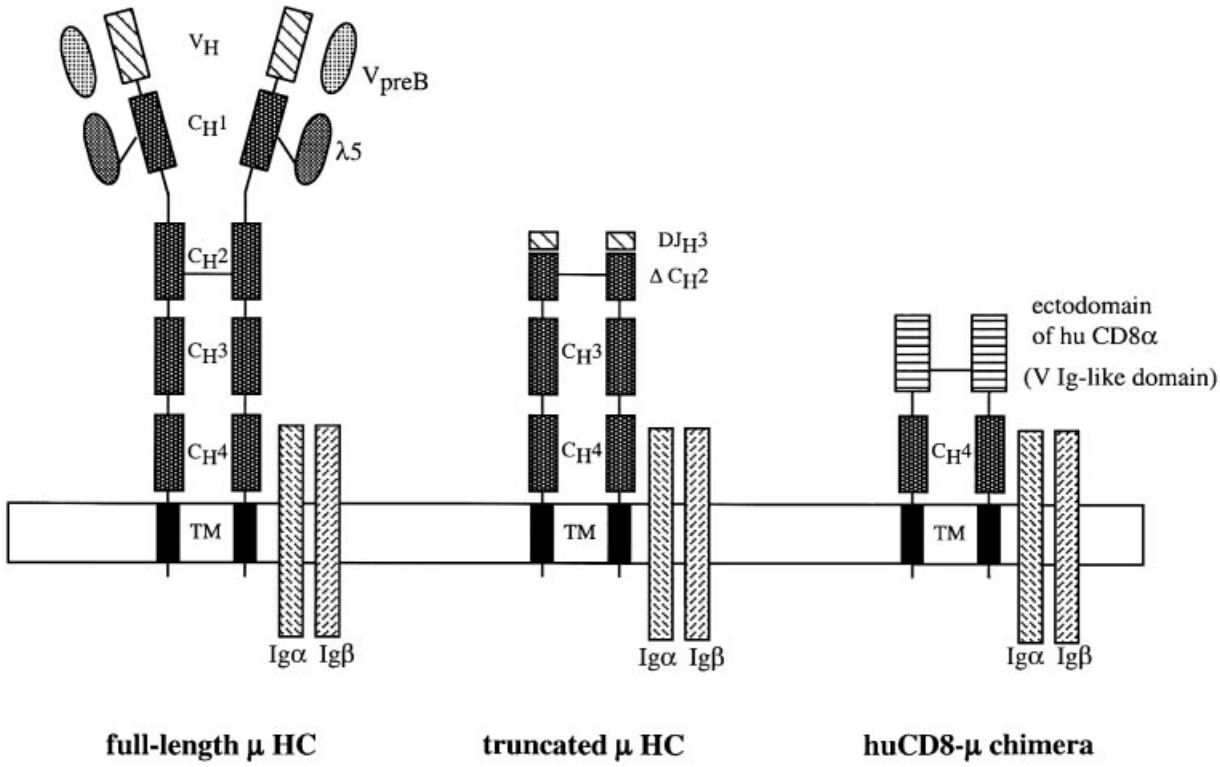

Fig. 1. Structure of pre-BCR and extracellular truncation mutants. (Left) The full-length μ heavy chain transgene encodes a productive variable domain (VDJ_H), four constant domains (C_H1–4) and a transmembrane domain (TM) followed by a 3-amino-acid residue cytoplasmic tail. This wild-type heavy chain associates with the surrogate light chains ($V_{pre\text{-}B}$ and λ5) and the signal transduction machinery (Igα and Igβ) to form a wild-type pre-BCR as depicted. The stoichiometry of the μ heavy chain homodimer to Igα:Igβ heterodimer is depicted as 1:1 in the pre-BCR as it has been shown to be in the mature BCR (32). (Middle) The truncated μ heavy chain transgene is encoded by a DSP2 D_H segment rearranged to a J_H3 segment (DJ_H3) ligated to a 5′ truncated C_H2 exon and the remainder of the heavy chain coding exons (31). Since this truncated μ heavy chain is missing most of its V_H and all of the C_H1 regions, it does not interact with $V_{pre\text{-}B}$ or λ5. (Right) The huCD8–μ chimera transgene is encoded by the extracellular region of human CD8α fused to the C_H4 exon and the remainder of the heavy chain coding exons (7). The huCD8α ectodomain is structurally similar to variable Ig domains and facilitates homodimerization of the chimeric chain through its native interchain disulfide bridge.

expression is independent of the V_H, C_H1, C_H2 and C_H3 domains of μ, and of the surrogate light chains.

Although the chimeric huCD8α protein lacks V_H and C_H1–3 domains, it remained possible that murine MHC class I molecules on the surface of bone marrow cells might serve as an artificial ligand for the huCD8–μ pre-BCR. In order to test this idea, we bred the huCD8–μ transgene onto the $\beta_2m^{-/-}$ genetic background which is deficient in MHC class I expression (16,17). β_2m deficiency resulted in a nearly 40-fold decrease in surface MHC class I expression (data not shown), but did not alter the ability of the huCD8–μ transgene to promote the pro- to pre-B transition (Fig. 4A). Given the failure of diminished MHC class I expression to effect transgenic B cell development, we conclude that MHC class I molecules are very unlikely to serve as ligand for the huCD8α-containing pre-BCR.

HuCD8–μ expression results in the allelic exclusion of endogenous IgH gene rearrangement

Expression of a transgenic wild-type heavy chain gene in developing B cells results in the allelic exclusion of endogenous IgH gene assembly (18,19). We analyzed the huCD8–μ transgenic mice to determine whether expression of this chimeric protein likewise results in allelic exclusion. We stained bone marrow cells from huCD8–μ transgenic and various control mice with anti-B220 and anti-μ antibodies. The anti-mouse μ monoclonal used in these studies does not recognize the huCD8–μ chimera. Flow cytometric analysis revealed that the huCD8–μ transgene suppressed surface expression of endogenous μ in the bone marrow (Fig. 5). Interestingly, we did observe significant endogenous heavy chain expression in splenic B cells from $RAG1^{+/-}$ huCD8–μ transgenic mice. Allelic exclusion mediated by the huCD8–μ transgene did not depend upon β_2m expression since we observed indistinguishable and greatly diminished IgM expression in both $\beta_2m^{+/-}$ and $\beta_2m^{-/-}$ huCD8–μ transgenic mouse bone marrow (Fig. 4B).

The ability of the huCD8–μ transgene to block surface expression of endogenous heavy chain in the bone marrow might occur at the level of heavy chain gene assembly or at the level of heavy chain protein expression. To elucidate the mechanism of this effect, we purified genomic DNA from transgenic and non-transgenic bone marrow, and performed a PCR analysis of V-to-DJ_H rearrangement (Fig. 6). While

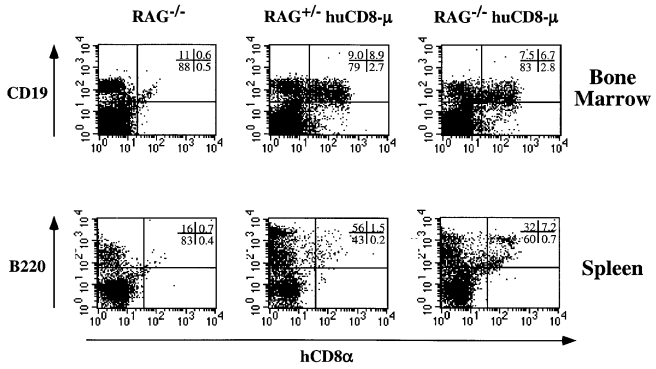

Fig. 2. Flow cytometric analyses of huCD8–μ transgene expression in bone marrow and spleen. Single-cell suspensions of whole bone marrow and spleen were prepared from RAG1-deficient ($RAG^{-/-}$), RAG1-heterozygous huCD8–μ transgenic ($RAG^{+/-}$ huCD8–μ) and RAG1-deficient huCD8–μ transgenic ($RAG^{-/-}$ huCD8–μ) mice. Cell surface expression of the pan-B cell markers CD19 or B220 was analyzed by flow cytometry after staining with the appropriate fluorochrome-conjugated antibody (see Methods). Cell surface expression of the huCD8–μ was detected by a mAb against human CD8α (hCD8α). Electronic gating based on forward and side scatter was employed to select for live lymphocytes. Quadrant statistics based on the live lymphocyte gate are indicated in the top right-hand corner of each FACS plot. (Upper panels) huCD8–μ transgene expression in the indicated bone marrow samples. (Lower panels) huCD8–μ transgene expression in the indicated spleen samples.

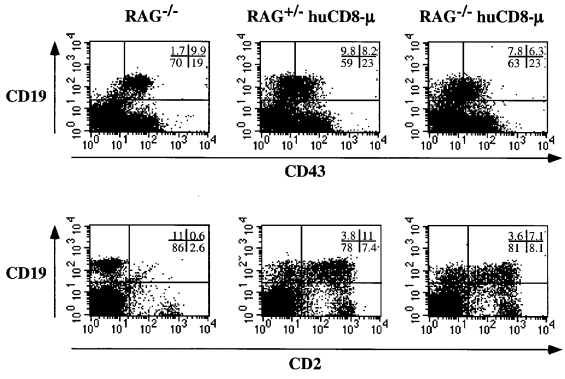

Fig. 3. Expression of the huCD8–μ transgene promotes the pro- to pre-B cell transition in the bone marrow. Single-cell suspensions of whole bone marrow were prepared from RAG1-deficient ($RAG^{-/-}$), RAG1-heterozygous huCD8–μ transgenic ($RAG^{+/-}$ huCD8–μ) and RAG1-deficient huCD8–μ transgenic ($RAG^{-/-}$ huCD8–μ) mice. (Upper panels) Flow cytometric analyses of samples stained with antibodies to CD19 and the developmentally regulated cell surface marker CD43. (Lower panels) Analyses of samples stained with antibodies to CD19 and the developmentally regulated cell surface marker CD2. Quadrant statistics are indicated in the top right-hand corner of each FACS plot.

huCD8–μ transgenic and control mice had similar levels of DJ_H-rearranged alleles, multiple individual mice from two distinct huCD8–μ founders showed a striking decrease in VDJ_H-rearranged alleles.

Assays which measure the abundance of rearranged alleles provide only an indirect measure of the targeting of the V(D)J recombinase to particular loci since cells containing specific rearranged alleles might undergo proliferation or death based on the structure of their encoded heavy chain protein. In order to more directly measure the effect of the huCD8–μ transgene on regulation of the V(D)J recombinase, we used a ligation-mediated PCR (LM-PCR) assay which detects double-stranded DNA breaks at recombination signal sequences (RSS) (Fig. 7A) (20). These RSS breaks are recombination reaction intermediates whose presence indicates active recombination at a given locus. As shown in Fig. 7(B), we were able to detect double-stranded DNA breaks 5′ of DJ gene segments in non-transgenic bone marrow (Fig. 7B, lane 4) and in bone marrow from mice which failed to express the transgene (Fig. 7B, lane 5). In contrast, we were unable to detect any 5′ of DJ breaks in bone marrow from five individual huCD8–μ-expressing mice from two different founders (Fig. 7B, lanes 6–10). These same transgenic bone marrow DNA samples did contain double-stranded DNA breaks 5′ of $J_\kappa 1$ (data not shown) and $J_\kappa 5$ (Fig. 7B, lanes 6–10). We conclude from this experiment that transgene expression results in the allelic exclusion of endogenous IgH gene rearrangement.

Discussion

The pre-BCR mediates a critical checkpoint in early B cell development, signaling success in the assembly of a gene encoding a functional, properly folded Ig heavy chain. The nascent pre-B cell undergoes several rounds of cell division, then exits the cell cycle and activates Ig κ locus rearrangement (1,2). A critical question in understanding the role of this receptor in mediating the pro- to pre-B transition is whether its function requires interaction with an extracellular ligand. It is possible that, distinct from other receptor systems, the pre-BCR is not designed to assess signals from the environment. Rather, its role may be to verify functional IgH gene assembly. Alternatively, it is possible that in addition to assessing the presence of a heavy chain, pre-B cells might undergo selection for the ability of the pre-BCR to recognize specific structures in their developing environment. These interactions might lead to deletion (or editing) of self-specific cells or positive selection of cells expressing potentially useful heavy chain structures.

Table 1. Absolute B cell numbers in $RAG^{-/-}$ and $RAG^{-/-}$ transgenic mice

Genotype	*n*	Total cells ($\times 10^5$)	$CD19^+$ cells ($\times 10^5$)	$CD19^+CD43^+$ cells ($\times 10^5$)	$CD19^+CD43^-$ cells ($\times 10^5$)
$RAG1^{-/-}$	3	103 ± 15	10 ± 3	10 ± 3	–
$RAG1^{-/-}$ × hCD8–μ	3	138 ± 6	18 ± 2	8 ± 1	10 ± 1
$RAG1^{-/-}$	3	181 ± 40	5.3 ± 2.5	5.3 ± 2.5	–
$RAG1^{-/-}$ × wt–μ	2	212 ± 60	7.5 ± 2.4	3.9 ± 2.9	3.5 ± 0.5

Values shown are the averages and SD from *n* mice (two hind legs per mice). Subpopulations were enumerated by FACS. For the top panel, mice were 6–8 weeks old. For the bottom panel, mice were 6 months old.

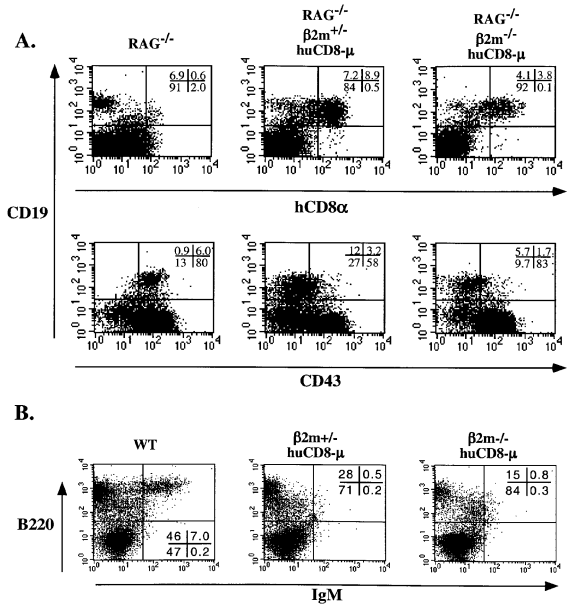

Fig. 4. Diminished MHC class I expression does not alter the ability of the huCD8–μ transgene to promote B cell development. (A) Bone marrow cells from RAG1-deficient ($RAG^{-/-}$), β_2m-heterozygous huCD8–μ transgenic ($\beta_2m^{+/-}$ huCD8–μ) and β_2m-deficient huCD8–μ transgenic ($\beta_2m^{-/-}$ huCD8–μ) mice were stained with antibodies to CD19, hCD8α and CD43. Quadrant statistics are indicated in the top right-hand corner of each FACS plot. (B) Bone marrow cells from $RAG^{+/+}$ wild-type (WT), β_2m-heterozygous huCD8–μ transgenic ($\beta_2m^{+/-}$ huCD8–μ) and β_2m-deficient huCD8–μ transgenic ($\beta_2m^{-/-}$ huCD8–μ) mice were stained with antibodies to B220 and IgM. Quadrant statistics are indicated in each FACS plot.

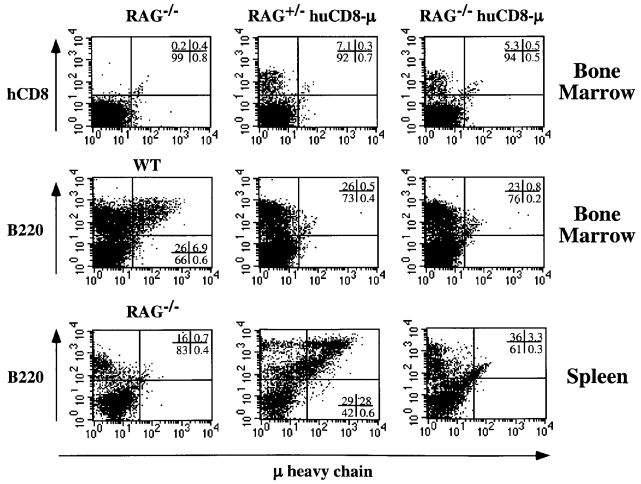

Fig. 5. Expression of the huCD8–μ transgene interferes with surface expression of IgM in the bone marrow but not in the spleen of transgenic mice. (Upper row) Bone marrow cells from RAG1-deficient ($RAG^{-/-}$), RAG1-heterozygous huCD8–μ transgenic ($RAG^{+/-}$ huCD8–μ) and RAG1-deficient huCD8–μ transgenic ($RAG^{-/-}$ huCD8–μ) mice stained with antibodies to hCD8α and IgM. (Middle row) Bone marrow cells from non-transgenic RAG1-heterozygous (WT), $RAG^{+/-}$ huCD8–μ and $RAG^{-/-}$ huCD8–μ mice stained with antibodies to B220 and IgM. (Bottom row) Spleen cells from non-transgenic RAG1-homozygous ($RAG^{-/-}$), $RAG^{+/-}$ huCD8–μ and $RAG^{-/-}$ huCD8–μ mice stained with antibodies to B220 and IgM. In the spleen of $RAG^{+/-}$ mice, a small percentage (2%) of the splenic cells co-express IgM and the huCD8–μ transgene; all of the splenic cells that express the huCD8–μ transgene also express surface IgM (data not shown).

The current work, as well as previous studies from our laboratory and others (5,6), has shown that the variable domain of the pre-BCR is dispensable for its function. These observations make it very unlikely that the variable domain of the pre-BCR mediates an essential positive selection step based on extracellular ligand binding. This, however, does not rule out the possibility that the pre-B cell repertoire is influenced by variable domain interactions. For example, it is possible that specific variable domain structures promote enhanced pre-B cell proliferation, resulting in an increased representation of that heavy chain in the immune repertoire. There is some evidence for such a positive selection step at the pre-B cell stage in the development of B1 B cells (21). In addition, Hardy *et al*. have noted distinct effects on survival of various heavy chain transgenes in fetal as compared to adult pre-B cells (22).

The present study was initiated in an attempt to understand how the pre-BCR might signal in the absence of its variable domain (6). The C_H2 and C_H3 domains of soluble Ig mediate binding of Ig to Fc receptors expressed on the surfaces of various cell types including B cells themselves. The experiments presented here rule out a necessary interaction between these domains on the pre-BCR and Fc receptor since the huCD8–μ transgene lacks the C_H2 and C_H3 domains. A recent report demonstrated that the B cell co-stimulatory molecule CD19 is capable of specific binding to IgM, but not to IgG (23). Therefore, it is possible that interaction between CD19 and the μ heavy chain within the pre-BCR might play a role in pre-BCR signaling. Our finding that almost the entire ectodomain of the μ chain is dispensable for pre-BCR signaling makes such a role for CD19 unlikely. Furthermore, CD19-deficient mice do not display an obvious defect in pre-BCR signaling (24,25). While a necessary interaction with the variable, C_H1, C_H2 and C_H3 domains of μ can be ruled out by our data, it remains possible that a putative pre-BCR ligand might interact with the ectodomains of either Igα or Igβ.

If the pre-BCR (and similarly the pre-TCR) does not require extracellular ligand engagement, how then does it signal? We favor a model presented in greater detail elsewhere in which signaling depends only on membrane localization of the pre-BCR, and the relative levels of protein tyrosine phosphatases and kinases (1). Surface transport of the pre-BCR, perhaps consequent to its localization in lipid rafts (26), results in the occasional interaction of kinases (Lyn, Fyn or Blk) and subsequent tyrosine phosphorylation of ITAMs on Igα, Igβ and signaling scaffolds leading to the recruitment of Syk. We propose that early in B cell development, the phosphatases which diminish the sensitivity of the BCR in mature cells are poorly expressed or not membrane associated, thus resulting in a milieu favoring receptor activity. Only minimal interaction between pre-BCR complexes is required to trigger the pre-BCR signal. Later in development, the level of phosphatases might increase, resulting in a requirement for BCR cross-linking in order to trigger a signal.

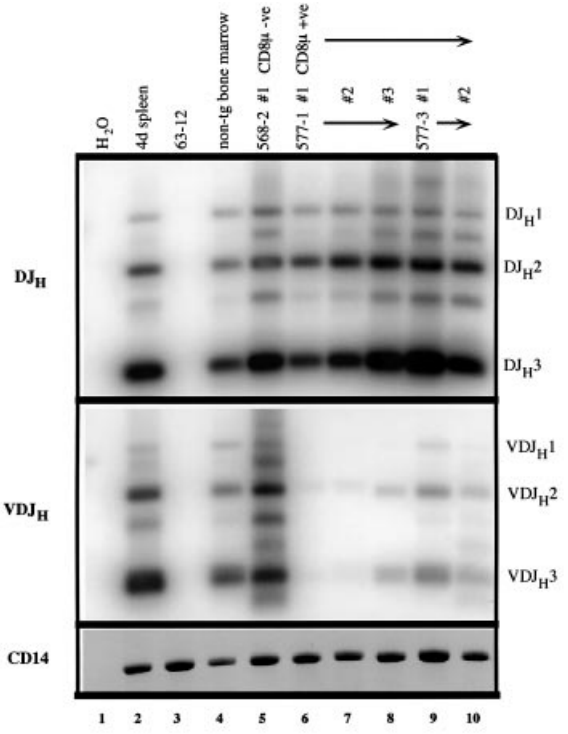

Fig. 6. Expression of the huCD8–µ transgene results in a decreased frequency of VDJ_H rearranged IgH alleles in transgenic mice. Genomic DNA samples were purified from cells described below and equivalent amounts were used as template in PCR assays. Water (lane 1) and genomic DNA from 63-12, a RAG2-deficient pro-B cell line (lane 3), serve as negative controls for rearrangements. Genomic DNA from spleen of a 4-day-old mouse (lane 2) and wild-type bone marrow from a 6-week-old non-transgenic littermate (lane 4) serve as positive controls for rearrangements. All genomic DNA samples from huCD8–µ transgenic mice were isolated from bone marrow cells (lanes 5–10). Mouse #1 from the founder line 568-2 (lane 5) carries the transgene, but does not express it at the protein level (data not shown). Multiple samples of genomic DNA from two independent lines of CD8–µ transgenic mice that did express the transgene were analyzed: three mice from founder line 577-1 (lanes 6–8) and two mice from line 577-3 (lanes 9 and 10). (Upper panel) Result of an assay for D-to-J_H rearrangements. (Middle panel) Result of an assay for V-to-DJ_H rearrangements. For the rearrangement PCR assays, products were separated by gel electrophoresis, blotted to nylon membranes, hybridized with radioactive probes specific for the products and revealed using a PhosphorImager. PCR amplifications of the CD14 gene were used to confirm that the quality and amount among the genomic DNA samples were similar (bottom panel).

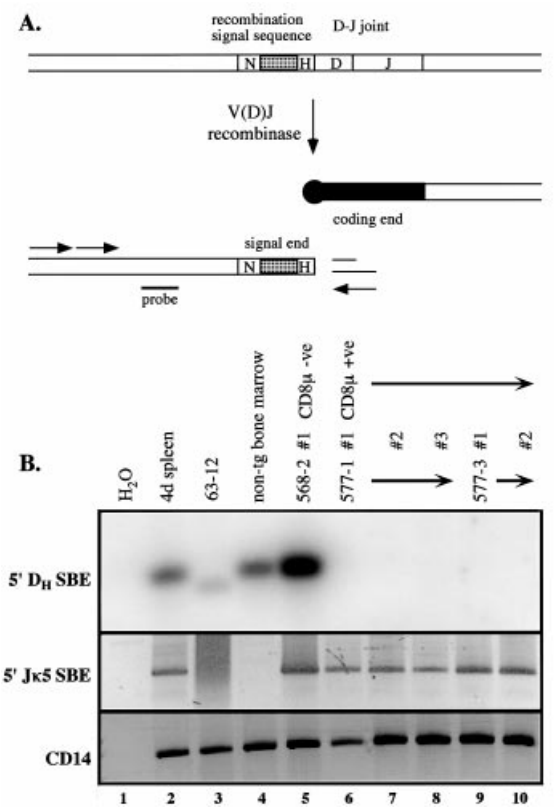

Fig. 7. LM-PCR analysis of RSS breaks in bone marrow of huCD8–µ transgenic mice reveals a block in V-to-DJ_H rearrangement. (A) A diagram showing the semi-nested LM-PCR assay used to detect 5′ of DJ_H signal broken ends (SBE) is shown. Broken DNA, consisting of a hairpin coding end and a blunt 5′-phosphorylated signal end (20), is an intermediate in the V(D)J recombination reaction. In a ligation reaction, linkers (depicted as an asymmetric pair of lines) will ligate to these blunt signal ends. These ligation products can be amplified by PCR using a linker-specific primer and locus-specific primers (arrows). Amplified signal ends can be detected by hybridization with a radiolabeled locus-specific DNA probe. (B) The samples of genomic DNA are identical to those indicated in the legend to Fig. 6 except that a linker-ligation step was implemented prior to semi-nested PCR. (Upper panel) Results of the LM-PCR assay detecting 5′ of DJ_H SBE (described in A). (Middle panel) Results of a different LM-PCR assay used to detect 5′ $J_κ5$ SBE. (Bottom panel) Direct PCR amplifications of the CD14 gene, a non-rearranging locus which serves as a control for DNA quality and quantity.

Observations regarding the biochemical function and developmental regulation of the B cell surface molecule CD22 are consistent with this hypothesis (27,28). CD22 is a transmembrane protein whose large cytoplasmic domain contains an ITIM which inducibly interacts with the protein tyrosine phosphatase SHP-1. CD22 is amongst the small set of membrane proteins which become tyrosine phosphorylated immediately after BCR cross-linking. Co-cross-linking of CD22 with the BCR strikingly inhibits the BCR signal (29). CD22 is developmentally regulated, with surface expression beginning at the late pre-B cell stage of development (28). Thus, the absence of CD22 upon initial pre-BCR expression might allow for signaling with extrinsic cross-linking, whereas later in development, the presence of CD22 must be overcome by a greater degree of BCR aggregation such as that induced by ligand engagement. Additional data consistent with our model was provided by an analysis of the effects of SHP-1 mutation (the motheaten *me*^{v} allele) on B cell tolerance. These studies showed that diminished phosphatase activity clearly alters the signaling threshold of the BCR (30).

It is important to consider whether mutant transgenes provide a valid model with which to study the structural requirements for the pre-BCR signal. It is possible that any particular mutant heavy chain might fold in such a way as to enhance its artifactual aggregation on the cell surface. Such aggregated protein might signal constitutively by clustering

associated Igα and Igβ molecules. Although we cannot rule out such a model, we believe it to be unlikely for two reasons. First, immunofluorescence analysis of a signaling transgenic mutant heavy chain protein shows a pattern of surface expression indistinguishable from that of wild-type heavy chain protein (6). Second, if the huCD8–μ protein was aggregating on the cell surface, we would expect it to undergo capping and be eliminated from the cell surface. This is clearly not the case. Ultimately, biophysical studies will be required to rule out this possibility.

Finally, the ability of the huCD8–μ transgene to enforce allelic exclusion is incomplete since $sIgM^+$ B cells do accumulate in the spleens of recombination competent transgenic mice (Fig. 5). This was surprising given the significant extent to which transgene expression decreases recombinase activity at the IgH locus as evidenced by the near absence of double-stranded DNA breaks 5′ of DJ_H alleles (Fig. 7). We suggest that the explanation of this paradox is that rare developing B cells which do generate an endogenous functional IgH gene rearrangement are the only developing B cells to survive since they can go on to rearrange the κ locus and generate a complete BCR. Recent work has shown that BCR expression is essential for B cell survival (31). These rare cells presumably accumulate in the spleen leading to the observed results.

Acknowledgements

The authors wish to acknowledge Dr Ashok Venkitaraman for sharing with us his huCD8–μ chimeric cDNA, and Dr Mark Soloski for β_2m-deficient mice and the anti-class I MHC antibody. This work was supported by a grant from the NIH (HL48702) and a Biomedical Science award from the Arthritis Foundation.

Abbreviations

β_2m	β_2-microglobulin
LM-PCR	ligation-mediated PCR
RSS	recombination signal sequence

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