Functional immunoglobulin transgenes guide ordered B-cell differentiation in Rag-1-deficient mice

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We have examined the regulatory role of the individual components of the immunoglobulin antigen receptor in B-cell development by transgenic complementation of Rag-1 deficient (Rag-1$^-$) mice. Complementation with a membrane μ heavy chain (μHC) gene allows progression of developmentally arrested Rag-1$^-$ pro-B-cells to the small pre-B cell stage, whereas the introduction of independently integrated μHC and κ light chain (κLC) transgenes promotes the appearance of peripheral lymphocytes which, however, remain unresponsive to external stimuli. Complete reconstitution of the B-cell lineage and the emergence of functionally mature Rag-1$^-$ peripheral B cells is achieved by the introduction of cointegrated heavy and light chain transgenes encoding an anti-H-2^k antibody. This experimental system demonstrates the competence of the μHC and κLC to direct and regulate the sequential stages of B-cell differentiation, defines the time at which negative selection of self-reactive B cells occurs, and shows that elimination of these cells occurs equally well in the absence of Rag-1 as in its presence. These data also support the hypothesis that Rag-1 directly participates in the *V(D)J* recombination process.

Cells of the mammalian immune system develop through an orderly process of differentiation, the end result of which is antigen-responsive B and T cells with individual immunoglobulin or T-cell receptor (TCR) surface receptors. In the murine bone marrow (BM), the sequential stages of B-cell differentiation have been classified into seven distinct fractions defined by surface antigenic characteristics (Hardy et al. 1991). The earliest large B-cell progenitors express CD43/S7 and differentially display B220/CD45R, HSA/30F1, and BP-1 surface markers and ordered rearrangements in the IgH loci (Ehlich et al. 1993; Li et al. 1993). Differentiation to the small pre-B-cell stage correlates with productive IgH rearrangements and the loss of CD43 expression.

In pre-B cells, the newly generated μ molecules reside predominantly in the cytoplasm (for review, see Rolink and Melchers 1991; Lassoued et al. 1993). A number of studies suggest that μ can also emerge on the surface of a transient BM subpopulation complexed with the two surrogate light-chain (SLC) molecules $\lambda 5$ and V_{preB} (Pillai and Baltimore 1987; Kerr et al. 1989; Karasuyama et al. 1993; Takemori et al. 1990; Tsubata and Reth, 1990; Cherayil and Pillai 1991; Misener et al. 1991; Nishimoto et al. 1991; Bossy et al. 1993; Lassoued et al. 1993). Surface expression of μ requires the additional appearance of the IgM auxilliary molecules Igα and Igβ (Nakamura et al. 1992). It has been suggested that at this particular checkpoint of B-cell development, μ has the dual role of promoting exclusion of the allelic IgH locus (Alt et al. 1984, 1987; Nussenzweig et al. 1987; Manz et al. 1988) and activation of transcription and rearrangement in the κ locus (Reth et al. 1987; Iglesias et al. 1991; Tsubata et al. 1992; Shapiro et al. 1993). Mutant mice lacking the ability to synthesize the membrane form of μ heavy chain (μHC) (μmT) fail to produce mature B cells and fail to exert exclusion at the allelic heavy-chain locus (Kitamura et al. 1991; Kitamura and Rajewsky 1992).

Following the expression of a functional μ, the fre-

quency of V_κ-to-J_κ rearrangements increases resulting in the production of a functional κlight chain (κLC) (Alt et al. 1987; Schlissel and Baltimore 1989; Schlissel et al. 1991a; Ehlich et al. 1993). The appearance of surface immunoglobulin $\mu + \kappa$ (IgM) receptor coincides with decreased levels of the SLC. Subsequently, cells display homing receptors (Clark and Lane 1991) and acquire competence to populate peripheral lymphoid organs. In contrast, B cells carrying immunoglobulin receptors with specificity for self-antigens undergo clonal deletion (Burnet 1959; Nemazee and Burki 1989a,b). In this way the organism eliminates inappropriate antigen receptor specificities soon after their genesis.

The genesis and diversity of antigen receptors is established by somatic rearrangements of their composite gene segments (Tonegawa 1983; Schatz et al. 1992). This process, termed $V(D)J$ recombination, employs ubiquitous components that include the scid (Bosma and Carol 1991), XR-1, and xrs-6 (Taccioli et al. 1993) activities, as well as the lymphoid-specific components Rag-1, Rag-2 (Schatz et al. 1989; Oettinger et al. 1990) and terminal deoxytransferase (TdT) (Landau et al. 1987; Gilfillan et al. 1993; Komori et al. 1993). Rag-1 and Rag-2 were identified by virtue of their ability to activate $V(D)J$ recombination of an artificial recombination substrate in fibroblast cell lines (Schatz et al. 1989; Oettinger et al. 1990). Their expression precedes antigen receptor rearrangement, and they are down-modulated by receptor engagement (Turka et al. 1991; Brandle et al. 1992; Ma et al. 1992). Mice homozygously deleted for either Rag-1 or Rag-2 show an arrest in lymphoid development at the point at which $V(D)J$ recombination of TCR or immunoglobulin loci would normally occur and are immunodeficient because of the lack of any functional B or T cells (Mombaerts et al. 1992a; Shinkai et al. 1992; L. Corcoran, E. Spanopoulou, and D. Baltimore, this study and unpubl.).

The early arrest of lymphoid development in Rag-1$^-$ mice does not allow the study of any possible functions of Rag-1 at later stages. By examining the influence of components of the immunoglobulin receptor on B-cell lineage progression in a Rag-1$^-$ background, their individual capabilities can be assessed and additional roles of Rag-1 beyond that of joining receptor gene segments could be uncovered. Thus, we have used transgenic complementation in the Rag-1$^-$ background to investigate the regulatory role of the individual components of the IgM receptor during the different stages of B-cell differentiation. We find that despite the absence of Rag-1, the μ plus κ chains of an authentic antibody can fully rescue Rag-1$^-$ B-lymphoid development, whereas μ alone can guide partial differentiation and a random μ plus κ combination allows only partial reconstitution.

Results

Rag-1 deficiency causes arrest of B- and T-cell development

To generate mice deficient for the Rag-1 protein (Rag-1$^-$), we constructed an insertion vector that disrupts the coding region of the Rag-1 gene at amino acid 330 (see Materials and methods). Examination of the immune system of Rag-1$^-$ homozygous mutant mice revealed the absence of any mature B or T cells in the periphery. In the thymus, T cells were arrested at the CD4$^-$/CD8$^-$ stage, prior to the point at which recombination of the TCR loci would normally occur (data not shown). The antigen receptor loci in Rag-1$^-$ lymphoid precursors remained in germ-line configuration despite the fact that they were transcriptionally active (data not shown). These data are in accordance with previous observations on Rag-1$^-$ (Mombaerts et al. 1992a) or Rag-2$^-$ (Shinkai et al. 1992) mice.

To analyze the stages of arrest of BM B-cell development, surface phenotypes were determined by FACS. For this purpose, seven fractions were distinguished according to previously defined criteria as presented in Table 1 (Hardy et al. 1991; Ehlich et al. 1993; Li et al. 1993). The B220$^+$/CD43$^+$ cells were subdivided into fractions A, B, C, and C' by their content of BP-1 and HSA (Fig. 1). In the Rag-1$^-$ mice, B-cell development was arrested in fraction C as indicated by the high levels of cells with HSA and BP-1 and the absence of CD43$^-$ cells that are the

Table 1. Stage-specific markers during B-cell differentiation in BM

Markers	Stage (fractions)						
	pro-B				pre-B (D)	immature B (E)	mature B (F)
	(A)	(B)	(C)	(C')			
CD43/S7	+ +	+	+	+	−	−	−
B220/CD45R	+	+	+	+ +	+ +	+ +	+ + +
HSA/30F1	−	+	+	+ + +	+ +	+	−/+
BP-1	−	−	+ +	+ +	+	−	−
$\lambda 5$	−	+ +	+ +	+ +	+	+	−
D_H to J_H	+	+ +	+ +	+	+	−	−
V_H to DJ_H	−	−	+	+ +	+ + +	+	−
V_κ to J_κ	−	−	−	+	+ + +	+	−

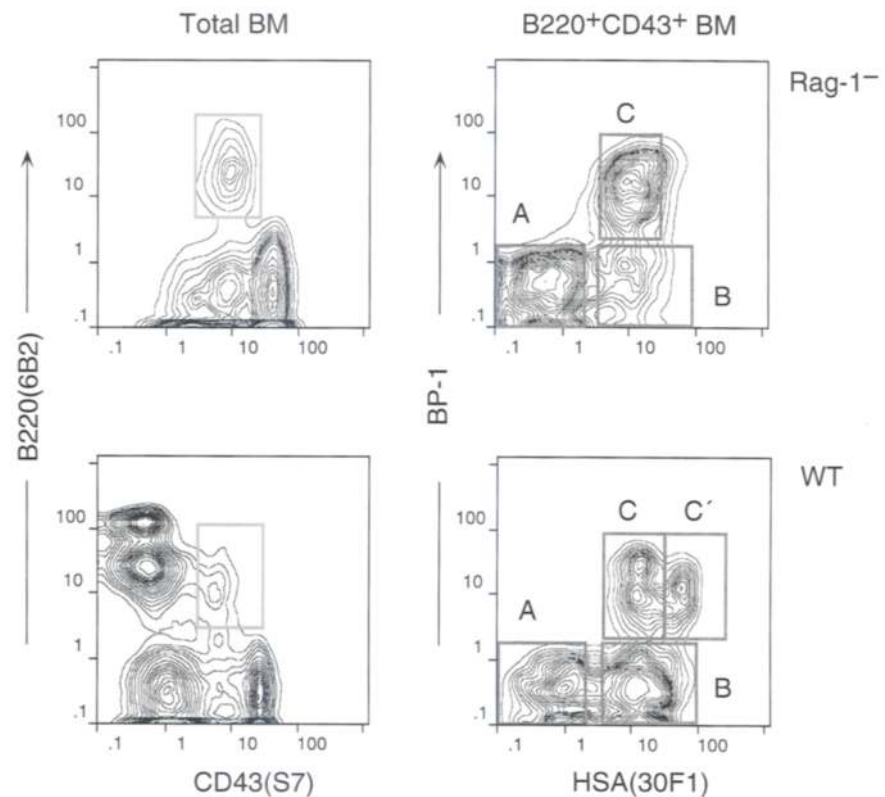The data are based on studies by Hardy et al. (1991), Ehlich et al. (1993), and Li et al. (1993).

Figure 1. Flow cytometric analysis of BM cells derived from Rag-1⁻ mice. Cells from BM of Rag-1⁻ or wild-type mice were resolved by four color FACS analysis for the surface markers B220/CD45R, CD43/S7, 30F1/HSA, and 30BP-1 (Hardy et al. 1991).

predominant ones in wild-type marrow (Fig. 1). Although fraction C was over-represented, cells failed to proceed to the very high HSA state of the C' fraction and fraction B was variable from animal to animal (Fig. 1, data not shown). Thus, B-cell development was arrested at the stage at which a functional μ would have been formed in normal mice. To examine whether the introduction of functional immunoglobulin receptors can override this developmental block, we complemented the Rag-1⁻ germ line with rearranged μ and κ transgenes.

*Generation and FACS analysis of Rag-1⁻/μ
and Rag-1⁻/$\mu\kappa$ mice*

To generate a Rag-1⁻/μ genotype, the Rag-1-deficient mice were crossed to transgenic mice expressing high levels of the membrane form of a human μHC under the regulatory influence of the IgH intronic enhancer. In these mice, the transgenic receptor promotes complete allelic exclusion of the endogenous IgH loci (Nussenzweig et al. 1987). No murine μ could be detected on the surface of wt/μ B cells (data not shown). A complete analysis of B-cell developmental fractions on a wild-type and Rag-1⁻ background is shown in Figure 2. Whereas lymphoid development was arrested in fraction C in Rag-1⁻ mice (Fig. 2, B1), the expression of a transgenic μHC alleviated this block and allowed B-cell development to proceed to the small pre-B cell stage (Fig. 2, B2, fraction D). The cells arrest in the stage at which recombination of the light-chain loci would normally occur (Table 1).

Surface expression of the transgenic μ protein remained undetectable by FACS analysis (Fig. 2, A2) despite its high levels in the cytoplasm (data not shown). Hence, the expression of the membrane form of μ was sufficient to shift cells from fraction C to D but little, if any, μ protein was evident on the cell surface.

To study the contribution of the κLC to early stages of B-cell development, Rag-1⁻/$\mu\kappa$ double transgenic mice were generated by backcrossing the Rag-1⁻/μ line to transgenic mice expressing a murine κLC regulated by the κ intronic enhancer. In the latter, the κ transgene is expressed at high levels in B cells and promotes allelic exclusion of the endogenous κ loci (Brinster et al. 1983; Storb 1987). In the Rag-1⁻/$\mu\kappa$ double transgenic mice, expression of the transgenic κ allowed differentiation of the small pre-B cells to the IgM⁺ immature B-cell stage (fraction E) (Fig. 2, A4 and B4). The transgenic human μ chain emerged on the surface complexed to the transgenic murine κ (Fig. 2, A4) and the cells were capable of exiting the BM and populating the peripheral lymphoid organs (Fig. 2, C4). Compared with wild type (Fig. 2, A/B8), however, lymphoid development in the Rag-1⁻/$\mu\kappa$ mice was still partly impaired. Specifically, there was a higher representation of pro-B cells (Fig. 2, cf. B4 with B8), the number of IgM⁺/B220^{high} cells in the BM was significantly lower and these cells did not proceed to the B220^{bright} stage (Fig. 2, cf. A/B4 with A/B5). In the Rag-1⁻/$\mu\kappa$ spleen, the total number of IgM⁺ cells was reduced and an intermediate subpopulation of B220⁺/IgM⁻ cells appeared (Fig. 2, cf. C4 with C5). The splenic
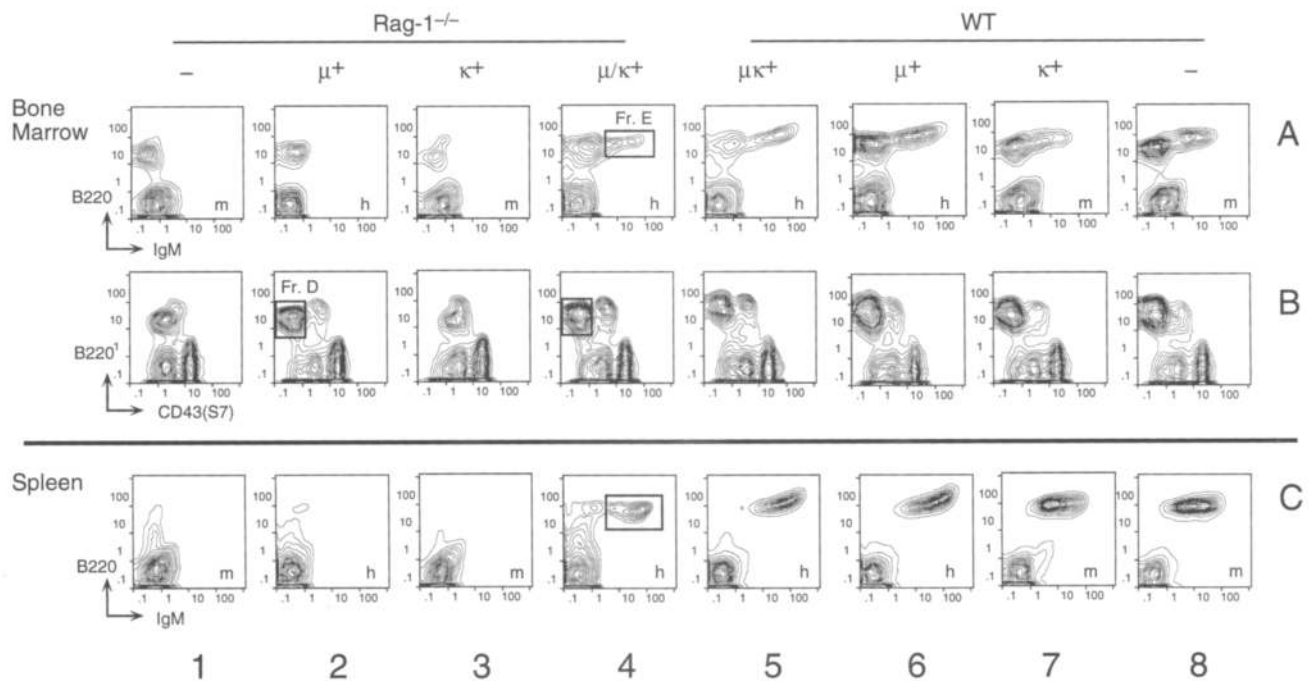
Figure 2. FACS analysis of the B-cell lineage in μ, κ, and $\mu + \kappa$ transgenic mice. BM and splenic lymphocytes isolated from wild-type (WT), Rag-1$^-$, and mice transgenic for rearranged transgenes expressing the membrane form of a human μHC (μ), or a murine κLC (κ), or both ($\mu + \kappa$), were resolved by four-color analysis for surface expression of B220/CD45R, CD43/S7, IgM, and HSA/30F1 (the latter is not shown) (Hardy et al. 1991). In lanes 1, 3, and 7, IgM refers to the murine (m) form, whereas in lanes 2, 4, 5, and 6, IgM refers to the human (h) transgenic protein. The human transgene encodes the membrane form of μHC and is regulated by the IgH intronic enhancer (Nussenzweig et al. 1987). The murine κLC transgene was isolated from the MOPC 21 myeloma and is regulated by the κ intronic enhancer (Brinster et al. 1983).

IgM$^+$ B cells expressed high levels of HSA (data not shown) and had only a modest response to lipopolysaccharide (LPS) stimulation (see below). In contrast, B-cell differentiation and maturation in the wt/$\mu\kappa$ mice more closely resembled the wild-type profile (Fig. 2, A/B/C8 and see below), although the total number of B220$^+$ cells in the BM is reduced. To examine whether a natural pair of HC and LC transgenes would provide complete reconstitution, a different series of Rag-1$^-$/IgM$^+$ mice were generated.

B-cell development in Rag-1$^-$ mice expressing an anti-H-2K^k D^k class I antibody

The new parental lines carried murine transgenes encoding an anti-H-2^k major histocompatibility (MHC) class I antibody (IgM/IgD) that recognizes K^k with moderate affinity, K^b weakly and fails to bind to H-2K^d expressing cells (Nemazee and Bürki 1989a; Russell et al. 1991). To determine the ability of the anti-H-2^k antibody to reconstitute the Rag-1$^-$ phenotype, we generated Rag-1-deficient mice transgenic (Tg$^+$) for the anti-H-2$^{k/b}$ antibody. The specificity of this antibody also allowed us to study the effect of Rag-1 on tolerance induction by providing a deleting H-2^b (^{b}) or permissive H-2^d (^{d}) MHC class I haplotype. An extensive analysis of the B-cell fractions in Tg$^+$ wild-type or Rag-1$^-$ mice is presented in Figure 3.

In the permissive Rag-1$^-$/Tg$^+$/^{d} mice, the BM was characterized by a lack of cells intermediate between the arrested pro-B cells of fraction C and fraction E cells, suggesting a transgene-driven acceleration of development (Fig. 3, cf. A/B4, with A/B1). In these mice, mature B cells extensively populated the periphery and expressed high levels of the transgene (Fig. 3, C4). Additionally, both IgM$^+$ and IgD$^+$ cells were generated (data not shown). Thus, the anti-H-2^k transgenic antibody directed B-cell lineage development differently from the $\mu\kappa$ hybrid receptor (cf. Fig. 3, A/B3/4 with Fig. 2, A/B4/5) allowing reconstitution to the mature B-cell stage. A similar phenotype was observed when the anti-H-2^k transgene was expressed in the wild-type background (Fig. 3, A/B/C2), in accordance to previous analyses (Nemazee and Bürki 1989a,b).

To examine whether MHC-mediated clonal deletion operates in the Rag-1$^-$ background, Rag-1$^-$/Tg$^+$/^{b} mice were generated. In these mice, no surface IgM was detected on the predominant B220^{high} subpopulation of the BM and these cells failed to proceed to the B220^{bright} stage (Fig. 3, A/B5). The absence of surface IgM$^+$ cells was also evident in the spleen where instead a novel subpopulation of B220$^+$/IgM$^-$/IgD$^-$ cells could be detected (Fig. 3, C5). Thus, negative selection operated efficiently in the absence of Rag-1. Similarly, expression of the transgenic antibody in the wt/H-2^b milieu led to
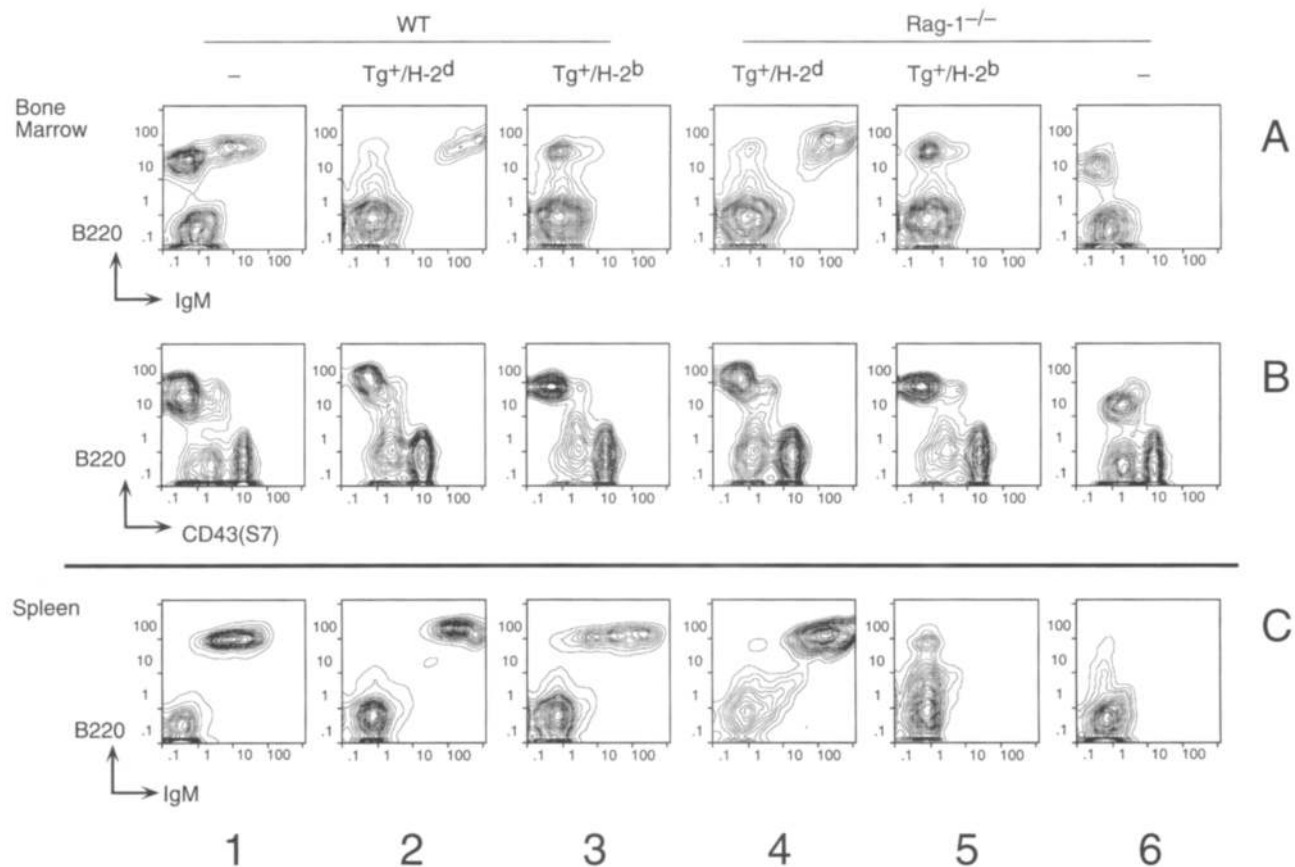
Spanopoulou et al.

Figure 3. FACS analysis of B-cell development in Rag-1-deficient mice transgenic for an anti-H-2^k MHC class I antibody (clone 3-83). Similarly to Fig. 1, bone marrow (BM) or splenic (SPL) B cells were resolved by four-color analysis for the surface markers B220, IgM, and CD43 and HSA (not shown). Genotypes were wild-type (WT), Rag-1 null (Rag-1$^-$), transgenic for an anti-H-2^k antibody (Tg$^+$) in either the deleting (H-2^b) or nondeleting (H-2^d) MHC class I haplotype. The heavy-chain transgenic genomic locus encodes the splicing variants for membrane and secreted IgH forms of both Cμ (IgM) and Cδ (IgD), in their natural configuration (Russell et al. 1991). The homologous light chain is encoded by its corresponding genomic fragment. The transgenes are transcriptionally regulated in part by their respective intronic and 3' enhancers and are cointegrated within the same chromosomal site. Therefore, it would be expected that onset of κ transcription is regulated in part by the IgH enhancers and vice versa.

complete elimination of all self-reactive B-cells in the BM and consequently to very low levels of IgM$^+$ cells (Fig. 3, A3). However, a high percentage of IgM$^+$ cells appeared in the spleens of these mice (Fig. 3, C3). This population was completely eliminated in the Rag-1$^-$ background (Fig. 3, C5) suggesting that these IgM$^+$ cells were generated by virtue of endogenous rearrangements (Tiegs et al. 1993).

Transcriptional analysis of B-cell-specific markers in transgenic mice

The relative homogeneity of the arrested BM cell populations in the Rag-1$^-$ background provided an opportunity to examine the properties of these cells. Initially, steady-state mRNA levels of certain lymphoid markers were determined using reverse transcriptase (RT)–PCR to quantitatively amplify specific transcripts (Schlissel and Baltimore 1989). The loci analysed included the IgH locus transcript μ, which persists throughout B-cell de-

velopment; the germ-line IgH transcript μ^o, which is expressed only in D-to-J recombining pro-B cells; and the LPS-inducible germ-line κLC transcript κ^o (see Schlissel et al. 1991b and references therein). No significant effects were seen in a wild-type background. As might have been expected from the phenotypic data, the μ transgene perturbed gene expression of the Rag-1$^-$ cells but the κ gene had no effect (Fig. 4). Transcript μ^o was expressed in the Rag-1$^-$ BM B cells and remained constant irrespective of the presence of the transgenic μ protein (Fig. 4). In contrast, transcript κ^o was expressed at low levels in the pro-B-cell stage (Rag-1$^-$), but assumed high levels in the presence of the transgenic μ protein and remained at high levels despite the coexpression of the transgenic κ protein (Fig. 4, κ^o lanes 5,6,7,8). Transcription of the surrogate light-chain molecule $\lambda 5$ was high in Rag-1$^-$ cells and decreased markedly in the presence of μ (Fig. 4, $\lambda 5$ lanes 5–8). Thus, in the transition from fraction C to D, μ allows the up-regulation of κ^o and down-regulation of $\lambda 5$ transcripts. In the same series
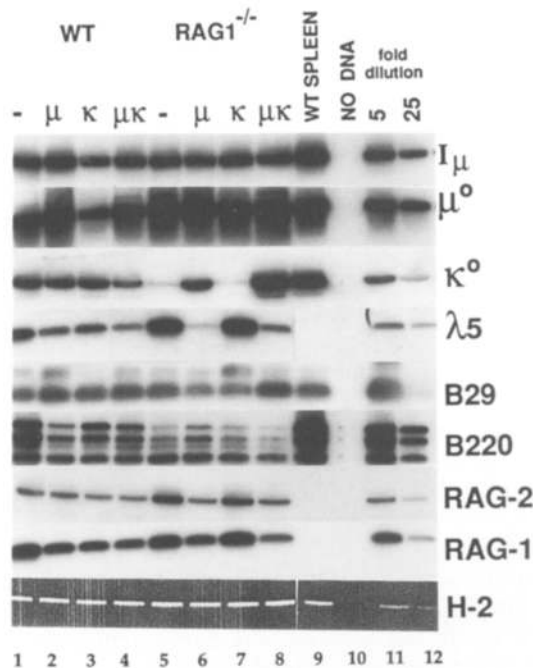
Figure 4. Transcriptional analysis of B-cell-specific markers in BM cells derived from human μ, mouse κ, and human μ plus mouse κ (hybrid receptor) transgenic mice. Cytoplasmic RNA was isolated from BM and used as a template for cDNA synthesis using random oligonucleotides. The cDNA products were analyzed by PCR using primers specific for the immunoglobulin loci germ-line transcripts Iμ, μ°, and κ°, (Schlissel et al. 1991b and references therein), the surrogate light chain molecule λ5, the immunoglobulin receptor-associated molecule B29, the B-cell-specific isoform of the surface marker B220, transcripts of Rag-1 and Rag-2 and, finally, the MHC class I transcript, which represents a ubiquitously expressed mRNA species. The final PCR products were detected by Southern blot hybridization except for the H-2 transcript, which is shown as a photograph of an ethidium bromide-stained gel. (Lanes *1–4*) Genotypes. (Lane *1*) No transgene (−); (lane *2*) transgene μ (human μ); (lane *3*) transgene κ (mouse κ); (lane *4*) transgene μ + κ (human μ plus mouse κ) all in a wild-type background; (lanes *5–8*) the same order of transgenic genotypes on a Rag-1⁻ background; (lane *9*) wild-type spleen; (lane *10*) no cDNA; (lanes *11,12*) products from dilutions of the wild-type spleen RNA (1/5 and 1/25, respectively) except in four cases: For the RAG-1 and RAG-2 products, lanes *11* and *12* represent dilutions of the RAG-1$^{-/-}$ BM sample; lanes *11* and *12* of λ5 represent dilutions of the wild-type BM sample. For the B29 product, because of a loading error, lane *11* corresponds to wild-type spleen and lane *9* shows the fivefold dilution of the sample. The Rag-1 primers amplify transcripts initiated 5′ to the disruption of the Rag-1 locus by the PGK *neo* gene.

of assays, the steady-state levels of transcripts B220, Iμ, and B29 remain unchanged among the Rag-1⁻, wild-type, or transgenic BM populations (Fig. 4). In contrast, Rag-1 and Rag-2 transcripts were decreased in the presence of the transgenes but remained easily detectable.

Stromal independence of reconstituted BM populations

Early stages of lymphoid development are characterized

by the interdependence of lymphoid progenitors and stromal epithelial cells (von Gaudecker 1986; van Ewijk 1991; Dorshkind 1990). Direct contact is required for early B-cell progenitor growth and survival, but secreted lymphokines, particularly interleukin-7 (IL-7), are sufficient to allow later cells to proliferate (Lee et al. 1989; Era et al. 1991; Hardy et al. 1991). The pro-B BM cell population (fractions B and C) proliferates selectively on a stromal cell layer (Hardy et al. 1987, 1991). To study the growth properties of these B220^{dull}/CD43$^+$ populations derived from Rag-1⁻ and Rag-1⁻/μ transgenic mice, they were purified by FACS sorting and cultured either directly on FLST2 fetal liver stromal epithelial cells or in a diffusion chamber to prevent direct contact with the stroma (Hardy et al. 1991). Cells were harvested after 4 days and counted for cell recovery. Wild-type pro-B cells proliferated without stromal support because they matured in culture to a contact independent phenotype (Fig. 5). In contrast, Rag-1⁻ pro-B cells proliferated on FLST2, as well as wild type, but did not grow in a diffusion chamber, indicating their absolute and continued contact dependence. However, expression of the transgenic μ at the pro-B/pre-B-cell stage was sufficient to alleviate stromal dependence. The Rag-1⁻/μ cells became contact independent and proliferated similarly to wild-type cells in the absence of any direct contact with FLST2 (Fig. 5), indicating that this pro-B-cell population contains a fraction of cells committed to differentiate from fraction C to fraction D.

Functional analysis of the transgenic peripheral lymphocytes

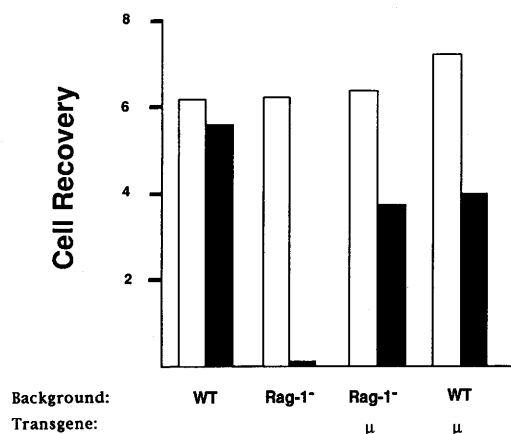The simultaneous introduction of rearranged μ and κ transgenes in the Rag-1⁻ genotype produced an overt

Figure 5. Stromal cell dependence of growth of sorted pro-B BM cells. B220^{low}/CD43$^+$/HSA$^+$ cells were isolated from the BM of mice of the indicated genotype and cultured either directly on the FLST2 stromal cell layer (Hardy et al. 1987) or in a diffusion chamber that prevents direct contact with stroma (Hardy et al. 1991). Cell viability was determined 4 days after cultures were established and is shown as fold increase. Genotypes are indicated below each bar. (Open bar) FLST2 contact; (solid bar) diffusion chamber.

shift in the differentiation of the arrested BM cells and enabled their exit to the periphery (Fig. 2, 4C, and Fig. 3, 4C). To study the response of the reconstituted peripheral lymphocyte cells to external stimuli, splenic B lymphocytes were induced with LPS. Cells from wild-type or Rag-1$^-$/Tg$^+$/^{d} mice readily responded to stimulation by LPS (Fig. 6). Rag-1$^-$/μκ splenic B cells, however, although variable, showed a reduced proliferation in response to LPS (Fig. 6), consistent with their lack of full maturation.

Subsequently, peripheral lymphocytes were tested for their ability to respond to cross-linking by anti-IgM, as measured by intracellular Ca^{2+} release (Fig. 7). Upon treatment, lymphocytes transgenic for either anti-H-2^k or μκ immunoglobulin receptors expressed in a wild-type background, showed a clear Ca^{2+} release signal (Fig. 7b,e). A similar response was observed in Rag-1$^-$ splenic lymphocytes that have been reconstituted with the anti-H-2^k transgenic receptor (Fig. 7f). In sharp contrast, however, the μκ hybrid receptor failed to fully induce B-cell maturation in the Rag-1$^-$ genotype, as evidenced by the lack of Ca^{2+} elevation (Fig. 7c).

The functional status of the reconstituted peripheral lymphocytes was also tested by assaying the phosphorylation of cellular proteins on tyrosine in response to anti-IgM (Gold et al. 1990, 1991). The results reiterated the Ca^{2+} release assays in that the Rag-1$^-$/Tg$^+$/^{d} splenic cells showed augmented protein–tyrosine phosphorylation (Fig. 8a, lanes 7,8) while cells with the hybrid μκ receptor were unresponsive (Fig. 8a, lanes 4–6). Finally, the difference between the two receptors in restoring the B-cell lineage of Rag-1$^-$ mice was reflected in the greatly

LPS response

Figure 6. Response of peripheral lymphocytes to LPS stimulation. B220$^+$ cells from the spleens of mice of indicated genotypes were isolated by cell sorting and incubated for 4 days in the presence of LPS. Subsequently, cells were harvested and counted. Wild-type cells expanded fivefold during the course of the 4-day incubation (bar labeled WT).

decreased levels of Igα and Igβ in the splenic lymphocytes of the Rag-1$^-$/μκ mice (Fig. 8b, lanes 15,16) compared with their Rag-1$^-$/Tg$^+$/^{d} counterparts, which expressed levels equal to those of wild type (Fig. 8b, lanes 7,8).
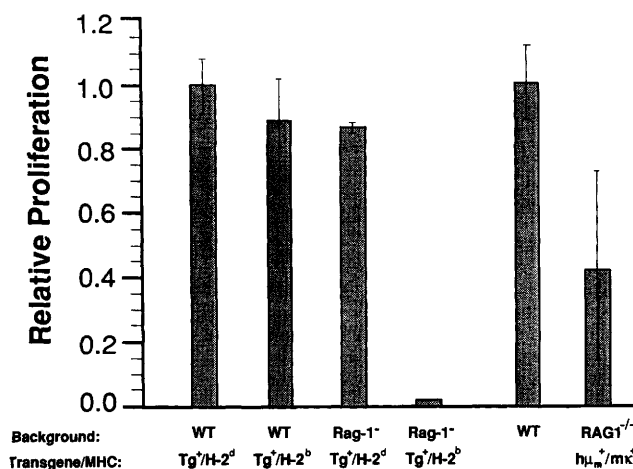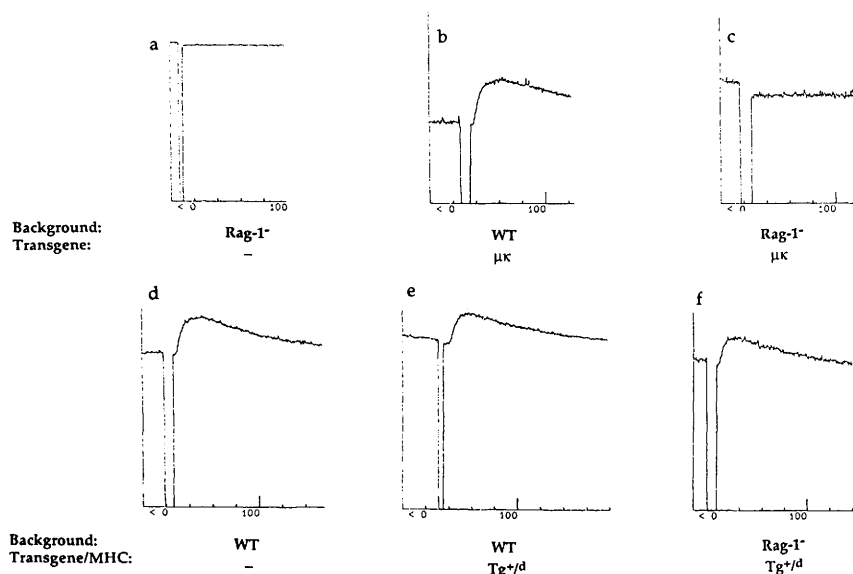
Discussion

The total absence of antigen receptor rearrangements in Rag-1-deficient mice emphasizes the indispensable role of Rag-1 in the V(D)J recombination process. In normal murine BM, Rag-1 and Rag-2 enable V(D)J recombination to initiate at the transition between fractions A and B (Table 1). Thus, the initial D-to-J_H rearrangements can be readily observed in fraction B while fraction C is characterized by V_H-to-DJ_H rearrangements (Hardy et al. 1991; Ehlich et al. 1993). The phenotypes of mice deficient in Rag-1 (this study) and Rag-2 (Shinkai et al. 1992) suggest that in the absence of receptor gene rearrangement, B-cell differentiation is abrogated after cells reach fraction C, as also observed in *scid* mice (Hardy et al. 1989). Given the extensive V(D)J recombinatorial activity in fractions B and C, the ability of Rag-1$^-$ and Rag-2$^-$ BM cells to proceed through these fractions and arrest in C suggests that the two proteins are involved in organizing or executing the V(D)J recombination reaction(s) rather than being stage-specific regulators of lymphoid development.

The mere expression of a functional immunoglobulin receptor allows complete reconstitution of the B-cell lineage in the Rag-1$^-$ background, implying that the lack of Rag-1 activity does not result in the absence of any developmental events that are crucial for B-cell differentiation. Similarly, the expression of a functional TCR in Rag-1- or Rag-2-deficient mice allows complete reconstitution of the T-cell lineage in these mice (Mombaerts et al. 1992b; Shinkai et al. 1993; E. Spanopoulou, C.J. Roman, W. van Ewijk, R. Hardy, P. Corbella, C. Mamoclouki, D. Moschofidis, L. Simpson, L.M. Corcoran, D. Kioussis, and D. Baltimore, in prep.). Additionally, the ability of the self-reactive B cells generated in the Rag-1$^-$/Tg$^+$/^{b} mice to undergo negative selection suggests that Rag-1 is not required for this process. The complete reconstitution of the B-cell lineage in the Rag-1$^-$/Tg$^+$/^{d} mice suggests that Rag-1 is not involved in the recombination of any as-yet-unidentified loci that could be critical to immunocyte differentiation. Finally, the lack of Rag-1 expression does not alter the expression of Rag-2, λ5, V$_{preB}$, CD43, HSA, BP-1, or germ-line immunoglobulin transcripts (this study; Mombaerts et al. 1992a; Shinkai et al. 1992). Thus, the only identified role of Rag-1 and Rag-2 in the lymphoid lineage is to ensure V(D)J rearrangement of the antigen receptor loci.

Expression of the membrane form of μ in the Rag-1$^-$/μ mice

Introduction of a functional μHC transgene, encoding the membrane-bound form, into the Rag-1$^-$ genotype enables the developmental transition of Rag-1$^-$ cells

Figure 7. Mobilization of intracellular Ca^{2+} following engagement of surface IgM. Splenic lymphocytes were isolated from mice of the indicated genotype, loaded with Indo-1, and subjected to IgM cross-linking with either anti-human μ or anti-mouse μ [both F(ab')₂]. The y-axis indicates the intracellular free calcium concentration; time is indicated in seconds on the x-axis.

from the pro-B- to the pre-B-cell stage, although no transgenic surface μ could be detected by FACS analysis. A similar phenotype has been described previously in the *scid*/μ transgenic mice, in which BM B cells proceed to the small pre-B-cell stage, whereas, no surface μ could be detected by FACS (Hayashi et al. 1990; Reichman-Fried et al. 1990, 1993). This could be the result of limitations in the level of detection by FACS . Low-level surface expression of the transgenic μ in Rag-1⁻/μ mice has

been revealed indirectly by probing for induction of tyrosine phosphorylation activity following the surface engagement of the μ protein (E. Spanopoulou, C.J. Roman, J. Friedrich, J. Cambier, R.R. Hardy, L.M. Corcoran, and D. Baltimore, in prep.).

The competence of the membrane μHC to alleviate the block imposed by the lack of *V(D)J* recombination has a close parallel in the T-cell lineage. Introduction of a functional TCRβ chain into either Rag-1- or Rag-2-
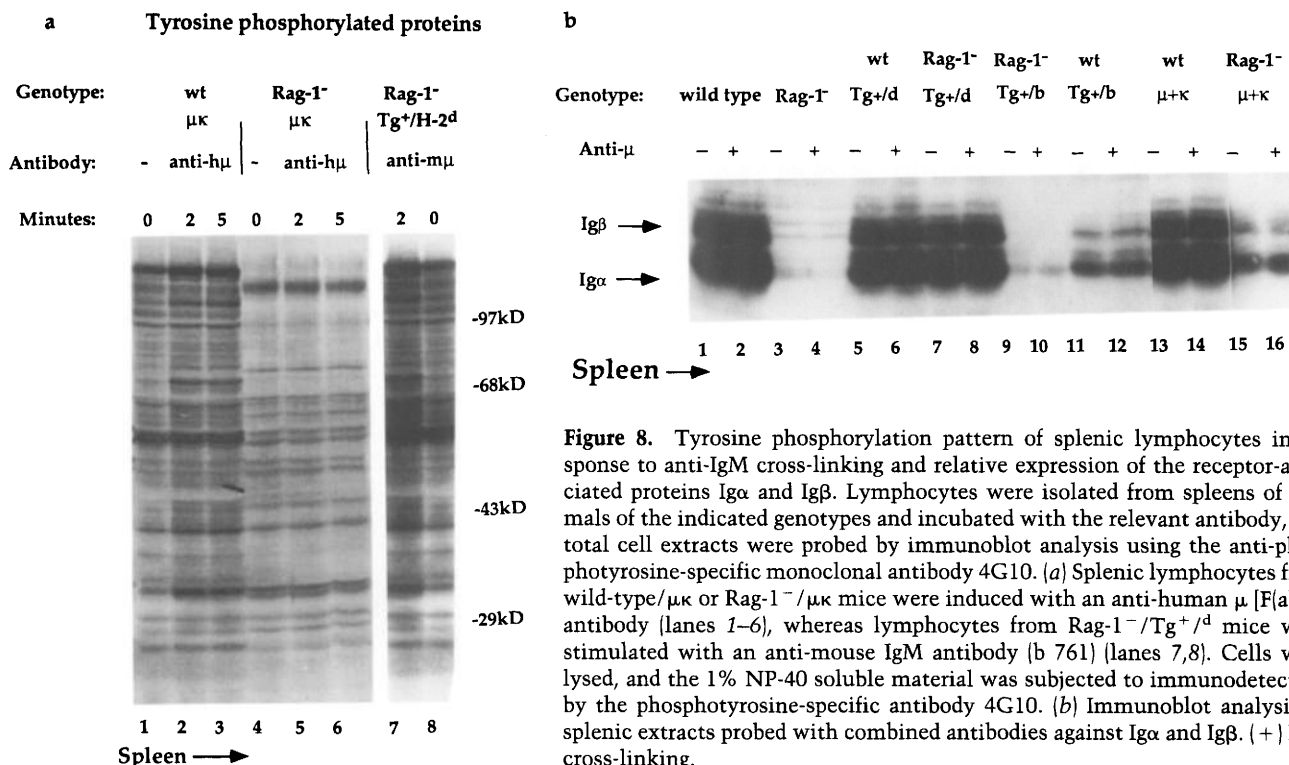

Figure 8. Tyrosine phosphorylation pattern of splenic lymphocytes in response to anti-IgM cross-linking and relative expression of the receptor-associated proteins Igα and Igβ. Lymphocytes were isolated from spleens of animals of the indicated genotypes and incubated with the relevant antibody, and total cell extracts were probed by immunoblot analysis using the anti-phosphotyrosine-specific monoclonal antibody 4G10. (a) Splenic lymphocytes from wild-type/μκ or Rag-1⁻/μκ mice were induced with an anti-human μ [F(ab')₂] antibody (lanes 1–6), whereas lymphocytes from Rag-1⁻/Tg⁺/ᵈ mice were stimulated with an anti-mouse IgM antibody (b 761) (lanes 7,8). Cells were lysed, and the 1% NP-40 soluble material was subjected to immunodetection by the phosphotyrosine-specific antibody 4G10. (b) Immunoblot analysis of splenic extracts probed with combined antibodies against Igα and Igβ. (+) IgM cross-linking.

deficient *scid* mice enables progression of the CD4$^-$/CD8$^-$ cells to the CD4$^+$/CD8$^+$ stage and expression of relatively small amounts of the β chain on the surface along with the CD3 auxiliary molecules γδε but not ζ (Kishi et al. 1991; Mombaerts et al. 1992b; Shinkai et al. 1993). The μHC and the TCRβ chain are similar in their competence to drive lymphoid differentiation, but one difference is the relative ease of detection of TCRβ on the surface of pre-T cells. Clearly, a limiting factor for μ or TCRβ to emerge on the cell surface is the expression levels of Igαβ or CD3 components, respectively (Nakamura et al. 1992; Levelt et al. 1993). An additional factor is suggested by the down-regulation of λ5 once a productive μ chain is expressed (Fig. 4). Thus, at least three components of the "potential" pre-B receptor complex appear to be present at very low levels in the pro-B/pre-B-cell populations consistent with the low levels of surface μ.

Introduction of membrane-bound IgM in Rag-1$^-$ mice

Ample surface expression of μ is permitted by the presence of a functional κLC. The regulatory role of the latter is evidenced in the Rag-1$^-$/μκ mice in which B cells are not held in fraction D but proceed to the B220^{high}/IgM$^+$ stage in the BM and acquire competence to emigrate and populate the peripheral lymphoid organs. However, these IgM$^+$ BM cells fail to reach the B220^{bright} stage observed in normal mice and peripheral transgenic lymphocytes retain a rather immature phenotype failing to respond to external stimuli. At present, we have no experimental explanation for this phenomenon. Both transgenes are particularly successful in pairing with the homologous endogenous chains (Brinster et al. 1983; Nussenzweig et al. 1987). In addition, the same human μHC is functionally active when paired with murine Igα and Igβ (Costa et al. 1992; Sanchez et al. 1993). Expression of the hybrid μ + κ receptor in a wild-type background does not impair B-cell maturation. This is possibly due to the expression of endogenous κLCs that form functional complexes with the transgenic μ. The data suggest that the hybrid receptor can become surface IgM but fails to mediate signaling in the final stages of differentiation. The maturation deficiency of the B cells in Rag-1/μκ mice, along with their deficit of Igα and Igβ, implies that surface IgM is involved in controlling at least two separable steps of B-cell differentiation, one of which is not operative in μκ mice but is operative in anti-H-2^k transgenics.

An interesting outcome of the studies on the Rag-1$^-$/Tg$^+$/^{b} mice is the observation that clonal deletion of the self-reactive B lymphocytes takes place equally efficiently in the absence of Rag-1 and T-cell help. Thus, B cells can undergo negative selection without having previously encountered T cells and in the absence of any *V(D)J* recombination activity. These data support previous suggestions that B-cell tolerance can be achieved in the absence of helper T cells (Bretscher and Cohn 1970; Golan and Borel 1971; Nemazee and Bürki 1989a). The phenotype of the deleting haplotype (Rag-1$^-$/Tg$^+$/^{b}) is

in accordance with the previously reported data that autoreactive B cells are counterselected by deletion (Nemazee and Bürki 1989a) and that both IgM and IgD isotypes of the anti-H-2^k receptor are being deleted (Russell et al. 1991).

The lack of endogenous immunoglobulin in the Rag-1$^-$/Tg$^+$/^{b} mice allowed us to examine the surface phenotype of the anti-H-2^b-positive BM cells under deletion conditions. Cells generated in the deleting haplotype reach the B220^{high} stage, whereas a permissive MHC enables the BM cells to progress to the B220^{bright} stage before they exit to the periphery (Fig. 3, cf. B4 with B5). Thus, elimination of self-reactive cells occurs at the transition from the B220^{high} to the B220^{bright} stage, consistent with previous suggestions about the timing of negative selection (Nemazee and Bürki 1989a,b; Nishimoto et al. 1991; Hartley et al. 1993).

The regulatory role of the μHC membrane form

A characteristic property of the membrane μ, demonstrated in pre-B-cell lines, is its ability to induce transcriptional activation of the κ locus (Iglesias et al. 1991) and subsequently a dramatic increase in the frequency of $V_κ$-to-$J_κ$ rearrangements in fraction D (Reth et al. 1987; Hardy et al. 1991; Iglesias et al. 1991; Ehlich et al. 1993; Li et al. 1993; Shapiro et al. 1993). This property is consistent with the model that transcription and recombination of the immunoglobulin loci follow an ordered pattern (Alt et al. 1987; Schlissel and Baltimore 1989; Schlissel et al. 1991a). However, the analysis of Rag-1$^-$ BM cells revealed that the κ locus is transcribed prior to the production of a μ, albeit at low levels. This discrepancy has also been observed in the μMT mice, in which BM cells show a low frequency of recombination in the κ locus despite the lack of any μ (Kitamura and Rajewsky 1992; Ehlich et al. 1993). Thus, in agreement with previous observations (Blackwell et al. 1989; Kubagawa et al. 1989; Schlissel and Baltimore 1989), our data suggest that the initial low levels of transcription and recombination in the κ locus are independent of the presence of the μHC. It is possible that initial κ transcripts give rise to functional κLCs (Kitamura et al. 1992). In contrast to this early phase, however, elevated κ transcription is dependent on μ as demonstrated by the phenotype of the Rag-1$^-$/μ BM cells. Therefore, the increase in transcription and frequency of recombination of the κ locus in fraction D appears to be a regulatory property of μ.

The transcriptional analysis of the Rag-1 and Rag-1$^-$/μ BM populations revealed the additional ability of μ to promote the down-regulation of λ5 in fraction D. This property of μ is in accord with the decreased levels of λ5/V_{preB} observed in the transition from fractions C to D in the BM of normal mice (Li et al. 1993) but is discrepant with previous observations on pre-B-cell lines (Iglesias et al. 1991; Kudo et al. 1992).

The regulatory role of μ at the pre-B cell stage is emphasized by the competence of the Rag-1$^-$/μ pro-B/pre-B cells to proliferate in the presence of IL-7 but independently of attachment to the BM stroma. These data are

similar to observations from wild-type and *scid* BM cells, indicating that early pro-B cells proliferate only in contact with the BM stroma but the expression of a functional μ at the transition from fraction C to C' enables entrance to the second, IL-7 responsive phase (Hayashi et al. 1990; Era et al. 1991; Reichman-Fried et al. 1993).

In conclusion, the phenotype of the Rag-1$^-$/μ mice underlines the multiple regulatory functions of membrane μ at the pre-B cell stage. In separate studies we were able to show that μ emerges on the surface of these cells albeit at low levels (E. Spanopoulou, C.J. Roman, J. Friedrich, J. Cambier, R.R. Hardy, L.M. Corcoran, and D. Baltimore, in prep.). Membrane μ is present at high levels in the cytoplasm, complexed to SLC or κ moieties (Pillai and Baltimore 1987; Takemori et al. 1990; Lassoued et al. 1993), apparently retained in the endoplasmic reticulum by BiP protein (Häas and Wabl 1983). Therefore, the question remains whether development of pre-B cells is directed by the μ resident on the cell surface and/or its cytoplasmic form and what is the exact functional significance of low levels of surface μ. A similar question is raised about the functional importance of low levels of TCRβ homodimers on the surface of pro-T cells (Mombaerts et al. 1992b; Shinkai et al. 1993).

The above studies show that B-cell development pauses at certain checkpoints, being dependent on the expression of the checkpoint determinant for transition to the next stage. Expression of these determinants, i.e., the membrane forms of μ or μκ, allows progression through the checkpoints and further differentiation.

Materials and methods

Targeted disruption of Rag-1

The mouse Rag-1 gene was isolated from transfected NIH-3T3 fibroblasts expressing Rag-1 activity (Schatz et al. 1989). The targeting vector carried genomic Rag-1 sequences (7.6 kb spanning the coding region) from the upstream *Eco*RV site to a 3' *Xba*I site). A cassette containing the PMC1 neomycin-resistance gene under the control of the PGK promoter, and carrying a polyadenylation signal (see Corcoran et al. 1993) was inserted into an internal *Eco*RV site in the Rag-1-coding sequence. The PGK *neo* insertion generated a frameshift mutation. A thymidine kinase cassette was inserted at the 5' end of the targeting vector to select against random integration.

J1 embryonic stem (ES) (SVJ129) cells were cultured on γ-irradiated (20Gy) primary embryonic (*Neo* resistant) fibroblasts as described (Corcoran et al. 1993). Twenty micrograms of linearized targeting vector was transfected into 8 × 10^6 ES cells by electroporation (400 V, 25 mF). Selection was initiated 48 hr later in the presence of 400 μg/ml of G418 with or without FIAU (0.2 mM). Resistant colonies were picked 8 days after selection, and homologous recombinants were identified by Southern analysis. Approximately 1/25 doubly resistant clones contained successful disruptions of the Rag-1 allele.

ES cell clones containing one RAG-1 mutant allele were injected into blastocysts of C57BL/6 mice and transplanted into the uteri of foster mothers. Several of the derived chimeric mice transmitted the mutant allele to their offspring, which were further backcrossed to establish mice homozygous for the mutation. Neonatal Rag-1$^{-/-}$ mice were genotyped by Southern

analysis to reveal a Mendelian pattern of transmission of the Rag-1 null allele.

Flow cytometry analysis, sorting, and culture conditions

Preparation of single cell suspension cells from the BM and subsequent immunostaining and FACS analysis, as well as sorting and culturing of the cells when required, were performed as described (Hardy et al. 1991) using the identical reagents. The human transgenic heavy chain was identified by an anti-human μ antibody (clone 145-8, Becton Dickinson), subsequently biotin conjugated.

RNA preparations and PCR assays

Preparation of total cellular RNA, reverse transcription, and subsequent analysis of the cDNA products by PCR were performed as described (Schlissel et al. 1991b) using the identical primers.

LPS stimulation proliferation assay

B220$^+$ cells from spleens of mice of indicated that genotypes were isolated by cell sorting and plated at 10^6 cells/ml (100 μl/well of a 96-well plate) in RPMI-1640 medium (supplemented with 5 × 10^{-5} M 2-mercapoethanol and 10% fetal calf serum) containing 10 μg/ml of LPS (Sigma). After 4 days, cells were harvested and counted.

Culturing on FLST2 stroma cells

Experimental conditions were as described previously (Hardy et al. 1987, 1991). Experimental cultures on FLST2 were carried out in 24-well plates (Nunc), using 1 ml of standard medium. Cultures were also performed by inserting diffusion chambers ("Millicell", Millipore Corp.) into wells containing pre-established FLST2 layers. Typically, 1 × 10^4 to 5 × 10^4 sorted cells were placed in each well.

Ca^{2+} mobilization assays

Loading of the cells with Indo-1 and measurements of intracellularly released Ca^{2+} were essentially performed as described (Costa et al. 1992). Indo-1-loaded cells were incubated at 37°C for 5 min, and transferred to a fluorimeter cuvette, and antibody was added to a final concentration of 15 μg/ml.

IgM cross-linking, preparation of cytoplasmic extracts, and Western analysis

Splenic lymphocytes were isolated without red blood cells and cultured at a density of 5×10^6/ml in RPMI/10% FCS/ β-mercaptoethanol at 37°C for 30 min prior to the addition of antibody. Anti-mouse Fab (Jackson Laboratory) or a rabbit polyclonal anti-human (Southern Biotechnology) was added to a final concentration of 25 μg/ml, and the suspended cells were incubated further for 5 min. Cross-linking was terminated by the addition of 12 ml of ice-cold PBS containing 1 mM orthovanadate, and the pelleted cells were lysed in 20 mM HEPES (pH 7.9), 100 mM KCl, 300 mM NaCl, 1% NP-40, 1 mM orthovanadate, 1 mM NaF, and 10 mM EDTA in the presence of several protease inhibitors. After sonication, extracts were separated on an 8% polyacrylamide gel and transferred onto nitrocellulose using 10 mM CAPS (pH 11.0), and 20% methanol. Filters were incubated in TBST (10 mM Tris at pH 8.0; 150 mM NaCl; 0.05% Tween 20) and blocked with either 2% BSA (for the anti-phos-

photyrosine antibody) or 2% milk for the Igα and Igβ antibodies, incubated with the primary antibodies overnight at 4°C, washed several times, incubated with the secondary antibodies for 30 min, washed again, and developed by alkakine phosphatase (Promega) or chemilluminescence (Amersham). The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from UBI.

Acknowledgments

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