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RAG2:GFP Knockin Mice Reveal Novel Aspects of RAG2 Expression in Primary and Peripheral Lymphoid Tissues

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Summary

We generated mice in which a functional *RAG2:GFP* fusion gene is knocked in to the endogenous *RAG2* locus. In bone marrow and thymus, RAG2:GFP expression occurs in appropriate stages of developing B and T cells as well as in immature bone marrow IgM$^+$ B cells. RAG2:GFP also is expressed in IgD$^+$ B cells following cross-linking of IgM on immature IgM$^+$IgD$^+$ B cells generated in vitro. RAG2:GFP expression is undetectable in most immature splenic B cells; however, in young RAG2:GFP mice, there are substantial numbers of splenic RAG2:GFP$^+$ cells that mostly resemble pre–B cells. The latter population decreases in size with age but reappears following immunization of older RAG2:GFP mice. We discuss the implications of these findings for current models of receptor assembly and diversification.

Introduction

Immunoglobulin (Ig) and T cell receptor (TCR) variable region genes are assembled in developing lymphocytes from germline variable (V), diversity (D), and joining (J) gene segments (Willerford et al., 1996). This V(D)J recombination reaction is effected by the same set of proteins in B and T lineage cells. The critical lymphocyte-specific components of the reaction are the recombination-activating proteins RAG1 and RAG2 that initiate V(D)J recombination by introducing double-stranded DNA breaks at the borders of Ig and TCR V, D, and J coding segments and adjacent recombination signal (RS) sequences (Gellert, 1997). Following cleavage by the RAG proteins, the liberated coding (and RS) ends are rejoined by a set of generally expressed proteins that carry out a nonhomologous end-joining reaction (Smider and Chu, 1997). The initiation of V(D)J recombination is tightly regulated via differential accessibility of antigen receptor loci and the lymphocyte-specific expression of *RAG1* and *RAG2* (Sleckman et al., 1996).

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In developing thymocytes, *RAG1* and *RAG2* are expressed first in the CD4$^-$CD8$^-$ double-negative (DN) compartment, which can be further subdivided based on the differential expression of CD44 and CD25 (Rodewald and Fehling, 1998). Past studies indicated that *RAG* expression initiates in the CD44$^+$CD25$^+$ stage and continues in the ensuing CD44$^-$CD25$^+$ stage, during which TCRβ, γ, and δ rearrangement commences (Wilson et al., 1994; Ismaili et al., 1996). Functional VDJβ rearrangement leads to expression of a pre-TCR composed of the TCRβ chain and the pTα protein, cellular expansion, and downregulation of *RAG* gene expression as cells progress to the CD44$^-$CD25$^-$ stage (Fehling and von Boehmer, 1997). Subsequently, TCRα gene rearrangement is initiated as $\alpha\beta$ lineage cells differentiate into CD4$^+$CD8$^+$ double-positive (DP) thymocytes and express high *RAG* levels (Guy-Grand et al., 1992; Wilson et al., 1994). *RAG* expression is downregulated a second time during the maturation of DP cells into CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ single-positive (SP) thymocytes, a process that can be mimicked in vitro by TCR cross-linking (Turka et al., 1991). $\gamma\delta$ lineage thymocytes also can be divided into immature and mature subsets based on expression of heat-stable antigen (HSA). Analogous to DP $\alpha\beta$ lineage thymocytes, HSA^{hi} $\gamma\delta$ thymocytes were reported to express *RAG1* and *RAG2* and to downregulate these genes in response to TCR cross-linking (Tatsumi et al., 1993).

In developing bone marrow (BM), *RAG1* and *RAG2* expression is first detected in pro–B cells undergoing sequential Ig heavy (H) chain D to J_H and V_H to DJ_H rearrangements (Willerford et al., 1996). Functional VDJ_H rearrangement leads to expression of a pre–B cell receptor composed of μH chain plus the V-pre-B and λ5 surrogate light chains, cellular expansion, and downregulation of *RAG* gene expression (Grawunder et al., 1995). *RAG* downregulation may prevent further V_H to DJ_H rearrangements on the second allele and thereby facilitate enforcement of IgH chain allelic exclusion. Subsequently, *RAG* genes are upregulated in later-stage pre–B cells, coincident with the onset of Ig light (L) chain gene rearrangement (Grawunder et al., 1995). Functional L chain expression leads to surface immunoglobulin M (sIgM) deposition and developmental progression to the immature B cell stage. In this context, sIgM cross-linking has been shown to downregulate V(D)J recombinase expression in various types of B cells (Ma et al., 1992; Hertz et al., 1998; Meffre et al., 1998). However, at least some immature BM B cells express *RAG* genes (Ma et al., 1992; Li et al., 1993; Grawunder et al., 1995), consistent with a capacity for receptor editing—a process whereby antigen receptor triggering by avid self-antigens leads to developmental arrest, secondary L chain gene rearrangements, and specificity changes (Hartley et al., 1993; Tiegs et al., 1993; Radic and Zouali, 1996; Chen et al., 1997; Pelanda et al., 1997).

Immature splenic B cells that have recently emigrated from the BM can be distinguished from their mature counterparts by lower B220 and IgD levels, higher HSA and IgM levels, and continued expression of the pB130–

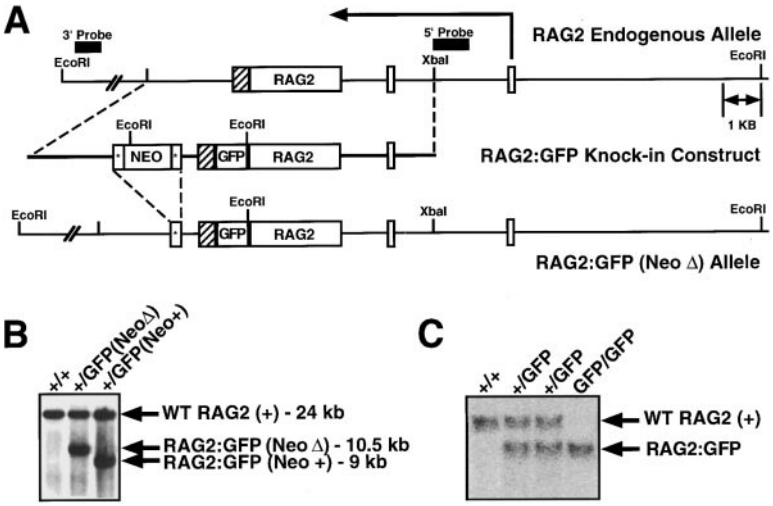

Figure 1. Generation of RAG2:GFP Knockin ES Cells and Mice

(A) A targeting vector was designed to replace the endogenous *RAG2* gene with the *RAG2:GFP* fusion gene (see text for details), employing regions 5′ and 3′ of *RAG2* to direct homologous recombination and a *loxP*-flanked PGK-*neo*^{r} gene. The endogenous *RAG2* allele is depicted with the *RAG2:GFP* knockin construct and a *RAG2:GFP* allele, following targeting and Cre-mediated deletion of the PGK-*neo*^{r} gene. Also indicated are the *RAG2* 3′ UTR (hatched boxes), *loxP* sites (starred boxes), and probes used for analysis of 5′ and 3′ homologous recombination (shaded boxes).

(B) Southern blot with the 3′ probe on EcoRI-digested DNA isolated from wild-type ES cells (+/+), ES cells targeted with the *RAG2:GFP* knockin construct [+/GFP (Neo+)], and the latter cells following Cre-mediated deletion of the PGK-*neo*^{r} gene [+/GFP (NeoΔ)].

(C) Southern blot with the 3′ probe on EcoRI-digested tail DNA of offspring from a mating of heterozygous RAG2:GFP knockin mice ($RAG2^{+/GFP} \times RAG2^{+/GFP}$).

140 marker (Allman et al., 1992, 1993; Rolink et al., 1998). These "transitional" B cells may also be distinguished from the immature BM B cells by their response to antigen receptor cross-linking. While immature BM B cells respond to BCR ligation by undergoing secondary L chain rearrangements, transitional B cells are highly sensitive to antigen-induced apoptosis in vivo (Carsetti et al., 1995). In addition, in vitro studies have shown that IgM^{lo} immature cells respond to BCR ligation by upregulating *RAG* genes and editing their receptors, while IgM^{hi} transitional cells respond to the same treatment by undergoing apoptosis (Melamed et al., 1998). This differential response of immature and transitional B cells to BCR cross-linking has been argued to result from the protective influence of the BM microenvironment (Sandel and Monroe, 1999). Collectively, these studies suggest that receptor editing may be the major tolerance mechanism of newly formed BM B cells, but as B cells mature and enter the spleen, they lose the capacity to edit and instead undergo apoptosis upon binding self-antigen.

The relative level of *RAG* gene expression in transitional splenic B cells has not been carefully examined. However, the finding of *RAG1* and *RAG2* expression in splenic B cells stimulated in vitro with LPS+IL-4 and in germinal center (GC) B cells of immunized mice suggested that at least some splenic B cells can be induced to reexpress or upregulate these genes (Han et al., 1996, 1997a; Hikida et al., 1996; Papavasiliou et al., 1997). *RAG*-expressing GC cells also can undergo secondary L chain gene rearrangements, possibly as a means of editing low-affinity receptors or receptors debilitated by somatic mutation (Kelsoe, 1999). Such GC cells could, in theory, represent pre–B/immature B cells in the spleen, as they share a number of characteristics with pre–B cells, including surrogate L chain gene expression, low B220 levels, and high HSA levels (Han et al., 1997b). A precedent for the existence of immature *RAG*-expressing B cells in the spleen is provided by studies of Igμ HC^{TG}/$BCL\text{-}2^{TG}$ $RAG2^{-/-}$ mice, which accumulate splenic pre–B cells within B cell follicles (Young et al., 1997). Similarly, activated Ras transgene expression in IgH chain–deficient B lineage cells also leads to accumulation of *RAG*-expressing, $sIgM^-$ splenic B lineage cells with rearranged L chain genes (Shaw et al., 1999). While the physiological significance of such mutant splenic B lineage cells is speculative, these studies demonstrate that pre-B-like cells are capable of leaving the BM in significant numbers and taking up residence in the spleen.

To examine further the developmental stages and physiological factors that lead to modulation of *RAG* expression, we have generated novel reporter mice in which a *RAG2:GFP* fusion gene replaces the endogenous *RAG2* coding exon.

Results

Generation of RAG2:GFP Knockin Mice

The *EGFP* gene (Clontech) was fused in-frame to the final codon of the *RAG2* gene. This fusion maintains the complete *RAG2* coding sequence, including the sequences that encode the phosphorylation site that directs cell cycle–specific degradation of the RAG2 protein (Li et al., 1996). The 3′ untranslated region (UTR) of *RAG2* was also retained 3′ of the fusion gene to provide polyadenylation signals and to allow for normal regulation. Thus, our reporter was designed to preserve as closely as possible the endogenous regulation of *RAG2* at both the transcriptional and posttranscriptional levels, including appropriate degradation of the reporter protein.

A targeting construct was engineered to replace the endogenous *RAG2* gene with the *RAG2:GFP* fusion gene, using genomic sequences 5′ and 3′ of the *RAG2* coding exon to direct homologous recombination and a neomycin resistance gene (PGK-*neo*^{r}) flanked by *loxP* sites for selection (Figure 1A). Targeted replacement of *RAG2* with the *RAG2:GFP* fusion gene and the *loxP*-flanked PGK-*neo*^{r} gene was carried out in TC1 (129SvEv) embryonic stem (ES) cells (Figure 1B). We subsequently

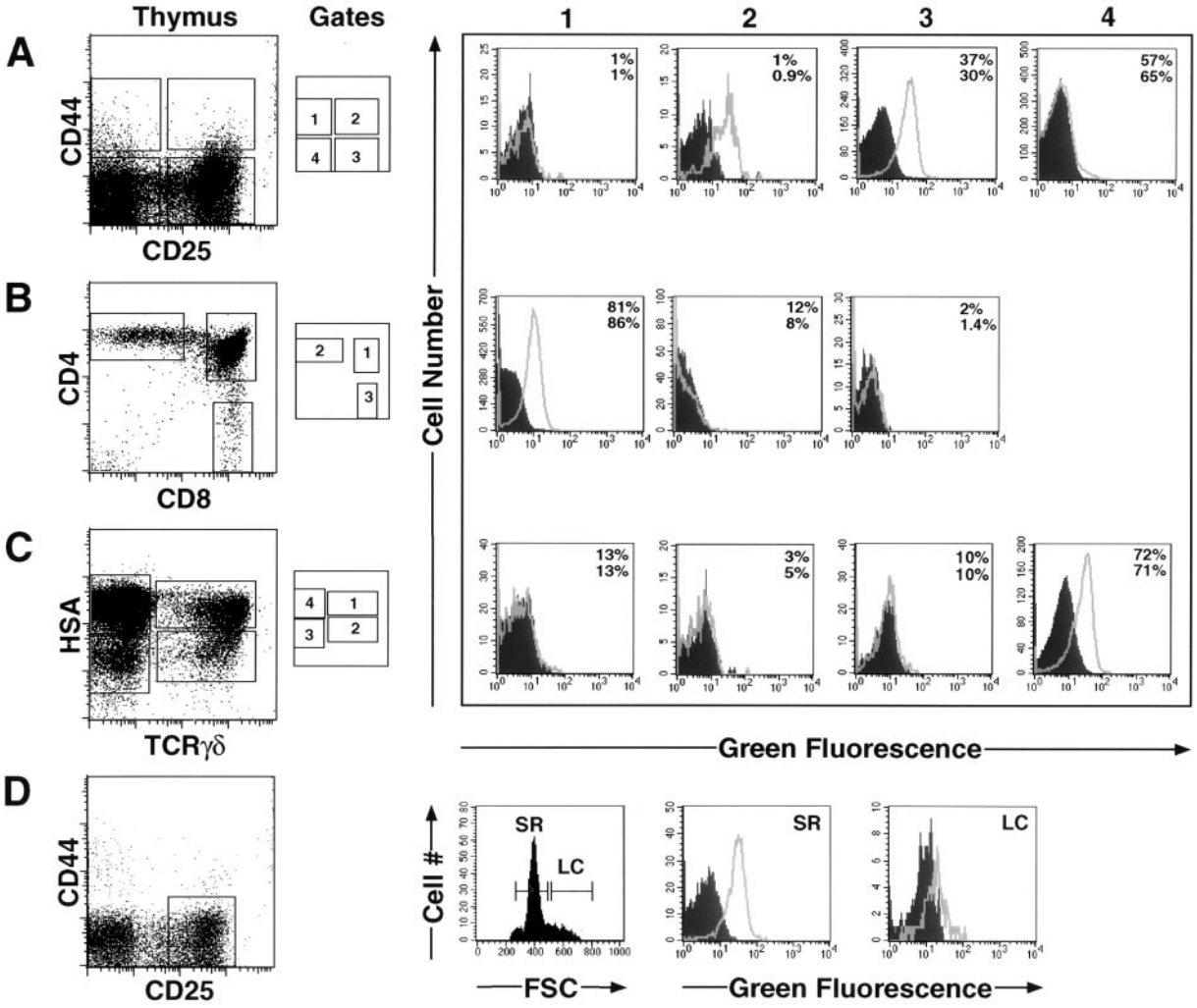

Figure 2. Expression of RAG2:GFP in Developing Thymocytes

FACS analysis was performed on thymocytes isolated from RAG2$^{+/+}$ (WT) and RAG2$^{GFP/GFP}$ (RAG2:GFP) mice after staining for the indicated cell surface markers. A representative FACS plot is shown for each set of markers, as there were no significant differences in phenotype between WT and RAG2:GFP thymocytes. For each of the gated populations (depicted by schematics), histograms for green fluorescence were generated. WT histograms (shaded) were overlayed with RAG2:GFP histograms (open); percentages of cells out of total live lymphocytes are shown for each population (top, WT; bottom, RAG2:GFP).

(A) Thymocytes were stained with phycoerythrin-conjugated (PE) anti-CD25, cychrome-conjugated (CyC) anti-CD44, and a cocktail of biotin-conjugated (bi) antibodies (bi-anti-CD4, -CD8, -B220, -MAC-1, and -GR-1), which were revealed with streptavidin (SA)-allophycoerythrin (APC). APC^{bright} cells were removed by electronic gating. CD44^{+}CD25^{-}, CD44^{+}CD25^{+}, CD44^{-}CD25^{+}, CD44^{-}CD25^{-} DN subsets were gated and analyzed for green fluorescence.

(B) Thymocytes were stained with PE-anti-CD4 and CyC-anti-CD8. CD4^{+}CD8^{+} DP, CD4^{-}CD8^{+} SP, and CD4^{+}CD8^{-} SP subsets were gated and analyzed for green fluorescence.

(C) Thymocytes were stained with PE-anti-TCRγδ, bi-anti-CD24 (revealed with SA-APC), and a cocktail of CyC antibodies (anti-CD4, -CD8, and -B220). CyC^{bright} cells were removed by electronic gating. TCRγδ^{+}HSA^{hi}, TCRγδ^{+}HSA^{lo}, TCRγδ^{-}HSA^{hi}, and TCRγδ^{-}HSA^{lo} subsets were gated and analyzed for green fluorescence.

(D) Thymocytes were stained as in (A). CD44^{-}CD25^{+} cells were gated and analyzed by forward scatter for small resting (SR) and large cycling (LC) subsets, which were assessed for green fluorescence.

used the Cre-*loxP* strategy (Gu et al., 1993) to isolate cells with Cre-mediated deletions of the PGK-*neo*^{r} gene (Figure 1B). These cells (RAG2$^{+/GFP}$) contain a single *loxP* site 400 bp downstream of the *RAG2* 3′ UTR on the *RAG2:GFP*–replaced allele. We used RAG2$^{+/GFP}$ ES cells to generate chimeras that were then bred to 129SvEv mice to obtain germline transmission of the *RAG2:GFP* gene. The heterozygous RAG2$^{+/GFP}$ offspring were subsequently crossed to generate homozygous RAG2$^{GFP/GFP}$, heterozygous RAG2$^{+/GFP}$, and wild-type RAG2$^{+/+}$ mice for analysis (Figure 1C).

RAG2:GFP Supports Normal V(D)J Recombination and Lymphocyte Development

As a preliminary test of the ability of the RAG2:GFP fusion protein to support V(D)J recombination, we transiently transfected a pCMV-*RAG2:GFP* expression construct, a *RAG1* expression construct, and the pJH200

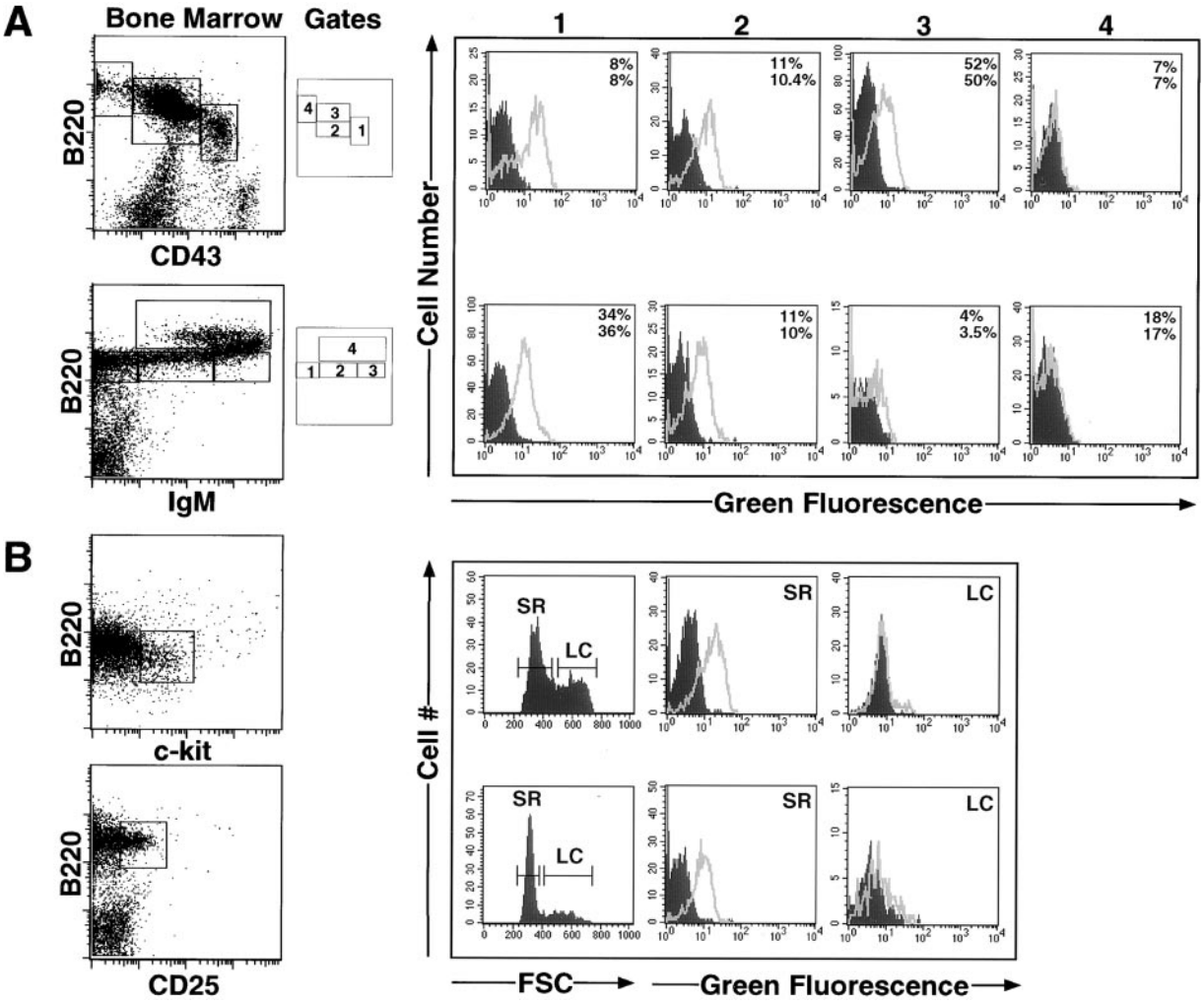

Figure 3. Expression of RAG2:GFP in Developing B Cells

(A and B) FACS analysis was performed on BM cells from RAG2$^{+/+}$ (WT) and RAG2$^{GFP/GFP}$ (RAG2:GFP) mice after staining with CyC-anti-B220 and either PE-anti-CD43, -IgM, -c-kit, or -CD25. Freshly isolated BM was used for all analyses except c-kit × B220, which was performed on BM cultured on the T220 stromal line for 1–2 weeks to enrich for c-kit$^+$ cells. A representative FACS plot is shown for each set of markers, as there were no significant differences in phenotype between WT and RAG2:GFP BM. For each of the gated populations in (A), histograms for green fluorescence were generated; gated populations in (B) were analyzed by forward scatter for small resting (SR) and large cycling (LC) subsets, which were subsequently assessed for green fluorescence. WT histograms (shaded) were overlayed with RAG2:GFP histograms (open); percentages of cells out of total live lymphocytes are shown for each population (top, WT; bottom, RAG2:GFP).

recombination substrate into CHO fibroblasts. In this assay, the RAG2:GFP protein promoted V(D)J recombination at levels comparable to wild-type RAG2 and did not interfere with wild-type RAG2 function when included in the same transfection (data not shown). In addition, lymphocyte development in RAG2:GFP heterozygous and homozygous knockin mice appeared normal, both with respect to developing populations as defined by surface marker expression and with respect to total thymocyte and splenocyte numbers (see below).

Regulated RAG2:GFP Expression in Developing Thymocytes

Based on CD44 and CD25 expression, RAG2:GFP homozygotes demonstrated a distribution of DN thymocyte subsets similar to that of wild-type mice (Figure 2A). Histogram analyses of green fluorescence demonstrated that RAG2:GFP is not expressed in early (CD44$^+$CD25$^-$) and late (CD44$^-$CD25$^-$) stage DN thymocytes but is expressed in the intermediate CD44$^+$CD25$^+$ and CD44$^-$CD25$^+$ subsets, the latter of which is characterized by the onset of TCRβ, γ, and δ rearrangements (Figure 2A). Furthermore, we observed that RAG2:GFP expression is downregulated between small resting CD44$^-$CD25$^+$ and large cycling CD44$^-$CD25$^+$ cells, consistent with the proposed point of TCRβ selection (Figure 2D; Hoffman et al., 1996). Analysis of DP and SP thymocyte subsets, as defined by CD4 and CD8 expression, also demonstrated a normal developmental pattern for RAG2:GFP homozygotes (Figure 2B). RAG2:GFP was highly expressed in CD4$^+$CD8$^+$ DP thymocytes, which undergo TCRα gene rearrangements, but was not expressed in CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ SP subsets (Figure 2B). Overall, RAG2:GFP expression in developing T cells faithfully recapitulates the developmentally regulated expression pattern of RAG2 in thymocytes

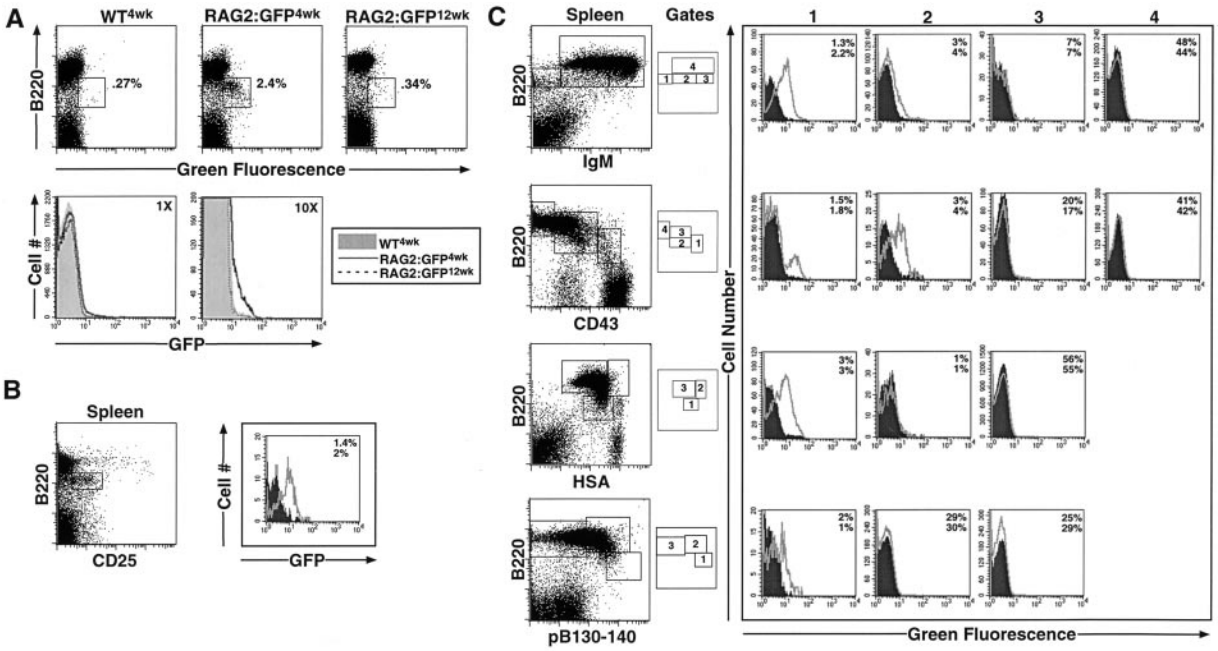

Figure 4. RAG2:GFP Is Expressed in a Small Population of Splenic B Cells

(A) Splenic cells from 4-week-old RAG2$^{+/+}$ (WT^{4wk}) and RAG2$^{GFP/GFP}$ (RAG2:GFP^{4wk}) and 12-week-old RAG2$^{GFP/GFP}$ mice were stained with CyC-anti-B220 and analyzed by FACS. The percentages of cells in the indicated B220^{lo}/GFP^{+} gate are shown. Histograms for green fluorescence were generated for each spleen and overlayed as depicted (1× and 10× views). GFP^{+} cells in the RAG2:GFP^{4wk} spleen are visible as a shoulder in the 10× view, in comparison to the WT^{4wk} and RAG2:GFP^{12wk} spleens.

(B and C) FACS analysis was performed on splenic cells from WT^{4wk} and RAG2:GFP^{4wk} mice after staining with CyC-anti-B220 and either PE-anti-CD25, PE-anti-IgM, PE-anti-CD43, PE-anti-CD24, or bi-493 (mAb against pB130-140—revealed with SA-PE). A representative FACS plot is shown for each set of markers, as there were no significant differences in phenotype between WT and RAG2:GFP spleen. For each of the gated populations (depicted by schematics for [C]), histograms for green fluorescence were generated. WT histograms (shaded) were overlayed with RAG2:GFP histograms (open); percentages of cells out of total live lymphocytes are shown for each population (top, WT; bottom, RAG2:GFP).

previously documented (Turka et al., 1991; Guy-Grand et al., 1992; Wilson et al., 1994; Ismaili et al., 1996), strongly indicating that the fusion protein is expressed and degraded similarly to wild-type RAG2.

We also analyzed the expression of RAG2:GFP in γδ thymocyte subsets. HSA^{hi} γδ^{+} thymocytes have been classified as immature and were reported to express *RAG2* mRNA, while HSA^{lo} γδ^{+} thymocytes have been classified as mature and lacking *RAG* expression (Tatsumi et al., 1993). In contrast, we did not find RAG2:GFP expression in either of these γδ thymocyte populations (Figure 2C). We did observe RAG2:GFP expression in HSA^{hi} γδ^{-} thymocytes, consistent with the expression of HSA in actively rearranging DN thymocyte subsets.

Regulated RAG2:GFP Expression in Developing BM B cells

B cell development in RAG2:GFP homozygous mice was normal, as determined by FACS analysis of surface expression of CD43, IgM, and B220 (Figure 3A). Furthermore, histogram analysis of the developmental subsets defined by these markers demonstrated that RAG2:GFP expression closely parallels previously reported *RAG* expression patterns (Figure 3A; Li et al., 1993; Grawunder et al., 1995). A high level of RAG2:GFP expression was found in CD43^{hi} pro–B cells, while a slightly lower level was found in CD43^{lo} pre–B cells. RAG2:GFP expression was not detectable in the B220^{hi}CD43^{-} BM subset composed of mature, recirculating B cells. Analysis of RAG2:GFP expression in BM populations defined by IgM and B220 showed that RAG2:GFP was expressed in most cells of both the IgM^{-}B220^{lo} and IgM^{+}B220^{lo} populations (Figure 3A). In addition, RAG2:GFP expression appeared inversely correlated with sIgM levels in BM B lineage cells, with green fluorescence intensity decreasing with increasing sIgM density. Although the majority of IgM^{hi}B220^{lo} cells had no detectable RAG2:GFP, we did observe a subpopulation of IgM^{hi}B220^{lo} cells with very low RAG2:GFP levels (Figure 3A).

In additional analyses, we observed that cells of both the c-kit^{+}CD25^{-} pre-B-I and c-kit^{-}CD25^{+} pre-B-II stages (Rolink et al., 1994) expressed RAG2:GFP (Figure 3B). In both the pre-B-I and pre-B-II stages, RAG2:GFP was highly expressed in the small resting but not the large cycling cells (Figure 3B), similar to the documented pattern of RAG2 protein expression in these populations (Grawunder et al., 1995). RAG2:GFP expression in these subsets was identical whether analyzed in freshly isolated BM samples or in BM cultures differentiated on the T220 stromal cell line. Our finding that RAG2:GFP expression faithfully reflects RAG2 protein expression in small and large pre-B-I and pre-B-II cells, in addition to small and large CD44^{-}CD25^{+} thymocytes, strongly suggests that the RAG2:GFP fusion protein is properly regulated during the cell cycle and targeted for degradation as cells exit G1 (Lin and Desiderio, 1994).

RAG2:GFP Is Expressed in a Small Population of Splenic B Cells in Young Mice

We examined expression of RAG2:GFP in splenic B and T cells to search for *RAG*-expressing B or T cells previously reported to be in the periphery (Han et al., 1997a; Papavasiliou et al., 1997; Hikida et al., 1998; McMahan and Fink, 1998). While we have not detected RAG2:GFP expression in peripheral T lineage cells, we have reproducibly identified a population of RAG2:GFP$^+$, B220$^+$ cells in the spleens of young mice; this population decreases in size with age and is not detectable after 8–10 weeks (Figure 4). These cells, which represent approximately 2%–3% of total splenic lymphocytes in 4-week-old RAG2:GFP mice, are readily apparent as a population of B220^{lo} cells (Figure 4A). We also have used antibodies against several other cell-surface markers to further characterize this population of GFP$^+$ B cells (Figures 4B and 4C). The majority are IgM$^-$B220^{lo}, although a few can be detected in the IgM^{lo}B220^{lo} subset. In addition, while there are small numbers of GFP$^+$ cells that are CD43^{hi}, most are CD43^{lo}. Analyses of splenic B cell populations defined by CD25, HSA, and pB130–140 demonstrate that most of the GFP$^+$ cells are CD25$^+$, HSA^{hi}, and pB130–140$^{+/hi}$. From this extensive FACS analysis, we conclude that the vast majority of the GFP$^+$ cells in the spleen are phenotypically identical to the pre–B and immature B cells observed in the BM of RAG2:GFP mice, with an additional small fraction of GFP$^+$ cells resembling pro–B cells.

RAG2:GFP Is Not Detectably Expressed in Splenic Transitional B Cells

When we compare RAG2:GFP expression in similar populations of BM and splenic sIgM$^+$B220^{lo} B cells, we observe that expression is significantly reduced in the splenic fractions versus BM fractions (compare panels in Figures 3A and 4C). As discussed above, most splenic GFP$^+$ B220^{lo} cells are sIgM$^-$, with a few having low levels of sIgM; we find no evidence of RAG2:GFP expression in sIgM^{hi}B220^{lo} or "transitional" B cells. To confirm that RAG2:GFP reporter expression in these cells truly reflected downregulation of *RAG* gene expression, we performed RT-PCR on sorted B220^{lo} populations of splenic B cells isolated from wild-type and RAG2:GFP (RAG2$^{+/GFP}$) mice (Figure 5A).

In accord with our FACS analyses, we find a high level of *RAG1* and *RAG2* expression, along with *λ5*, in IgM$^-$B220^{lo} fractions, a very low level of *RAG* expression in IgM^{lo}B220^{lo} cells, and negligible levels in IgM^{hi}B220^{lo} fractions (Figure 5B). Specifically, levels of *RAG1* and *RAG2* transcripts are reduced ~25-fold or more in the sIgM^{lo} and ~100-fold or more in the sIgM^{hi} fractions as compared to levels in the sIgM$^-$B220^{lo} fraction (Figure 5C). The very low–level *RAG* expression in the sIgM^{hi} fraction might reflect low-level *RAG* expression in many of these cells or, more likely, the presence of a few cells that express high *RAG* levels, possibly as a result of a small number of contaminating sIgM$^{-/lo}$ cells. In either case, we conclude that splenic transitional B cells do not express appreciable *RAG* levels and that the *RAG* expression in the splenic B cell compartment is derived primarily from pre–B/immature B cells.

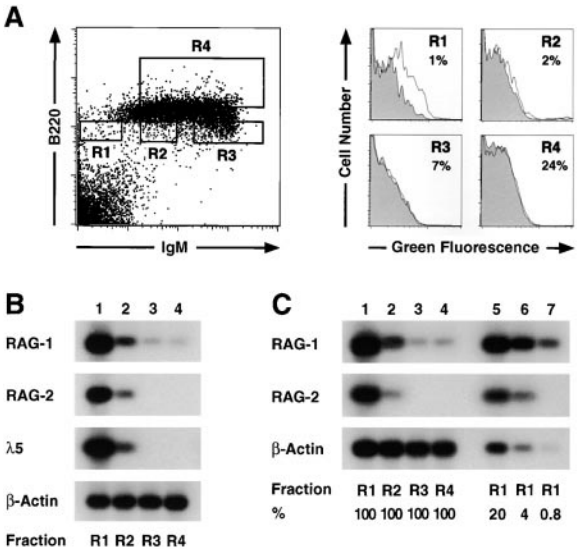

Figure 5. Expression of RAG2:GFP Is Downregulated in Splenic Transitional B Cells

(A) Four fractions (R1–R4) of splenic B cells from RAG2$^{+/GFP}$ and RAG2$^{+/+}$ mice were sorted based on surface expression of B220 and IgM. Histogram overlays depict the relative levels of green fluorescence in RAG2$^{+/+}$ (shaded) versus RAG2$^{+/GFP}$ (open) fractions. The indicated percentages of RAG2$^{+/GFP}$ cells in each fraction are calculated out of total live B220$^+$ lymphocytes. Purity of all sorted fractions was ≥95% as assessed by FACS reanalysis.

(B) RNA was purified from sorted RAG2$^{+/GFP}$ fractions R1–R4 and used for RT-PCR analysis of *RAG1*, *RAG2*, and *λ5* mRNA expression. *β-actin* was used as a control to ensure equivalent amounts of mRNA. Equivalent results were obtained from sorted RAG2$^{+/+}$ fractions (data not shown).

(C) Serial 5-fold dilutions of fraction R1 cDNA were made and used for RT-PCR analysis of *RAG1* and *RAG2* mRNA expression in parallel with undiluted R1–R4 cDNA. *β-actin* was used as a control to ensure equivalent amounts of mRNA.

RAG2:GFP Is Expressed in a Subset of Splenic B Cells following Immunization

To determine whether RAG2:GFP–expressing cells could be found in the spleens of older mice following immunization, we immunized 10- to 12-week-old RAG2:GFP (RAG2$^{GFP/GFP}$) and wild-type 129SvEv mice with chicken γ-globulin substituted with the (4-hydroxy-3-nitrophenyl)acetyl hapten (NP-CGG). Subsequently, we analyzed the mice for the presence of splenic GFP$^+$ cells at days 8 and 16 following immunization in comparison with unimmunized (naive) littermates. Naive RAG2:GFP mice did not have detectable GFP$^+$ splenic B cells. Strikingly, at day 16, but not day 8, following immunization, we observed a significant population of splenic GFP$^+$ B cells. These cells, like the GFP$^+$ cells in young RAG2:GFP mice (Figure 4A), are readily apparent as a population of B220^{lo} cells (Figure 6A). In addition, these GFP$^+$ cells express little or no sIgM and have uniformly high levels of the immature B cell marker pB130–140 (Figure 6B). The B220^{lo}/pB130–140$^+$ population enriched for GFP$^+$ cells was not present in spleens of naive mice or those analyzed at day 8 following immunization. In previous reports, *RAG* expression has been documented in a population of splenic B cells identified by the GL-7 marker (Han et al., 1997a). We find that of the B220^{lo}/

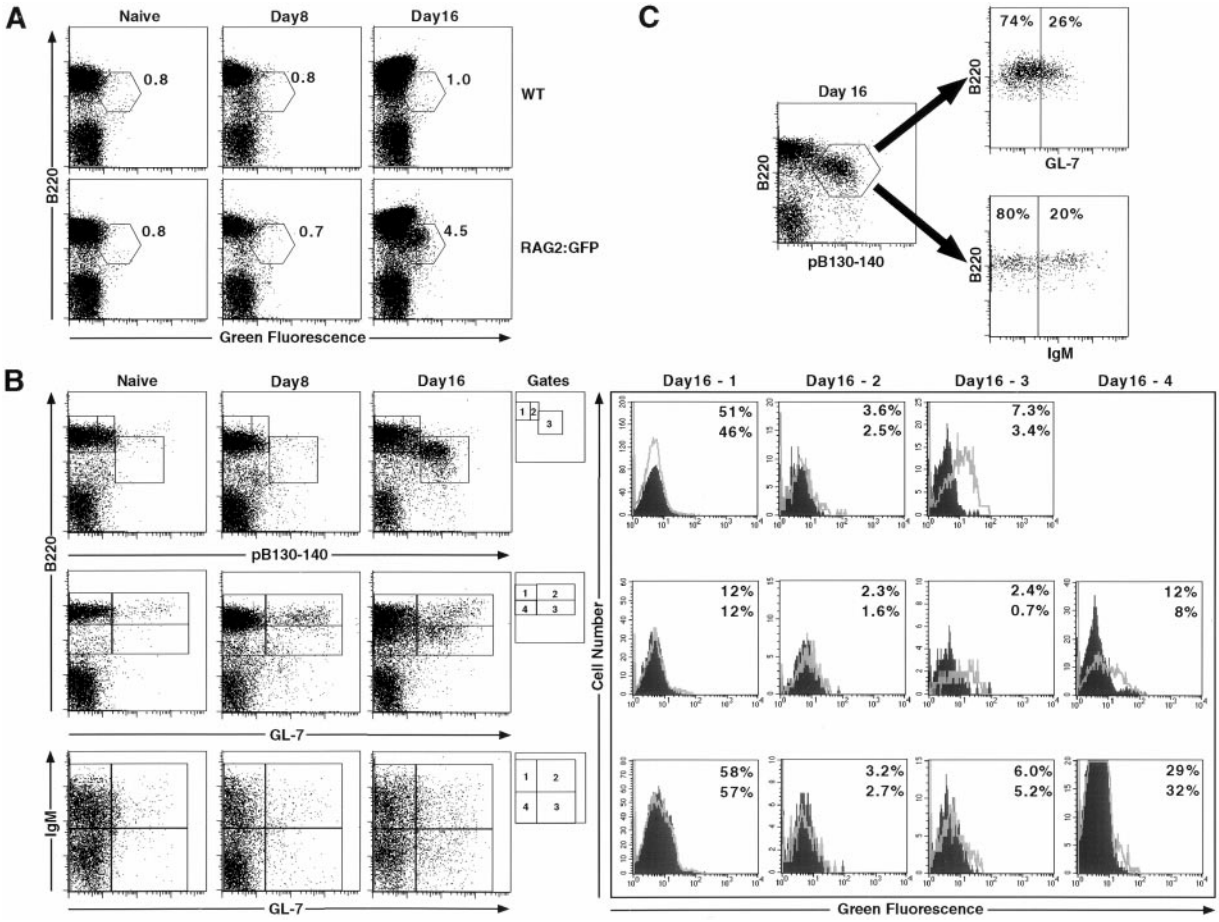

Figure 6. RAG2:GFP Is Expressed in a Subset of Splenic B Cells following Immunization

(A) Splenic cells from naive RAG2$^{+/+}$ (WT) and RAG2$^{GFP/GFP}$ (RAG2:GFP) mice and from RAG2$^{+/+}$ and RAG2$^{GFP/GFP}$ mice at 8 and 16 days after immunization with NP-CGG ("day 8" and "day 16") were stained with PE-anti-B220 and analyzed by FACS for expression of RAG2:GFP. The percentages of cells in the indicated B220^{lo}/GFP^{+} gate are shown. Several 10- to 12-week-old mice of each phenotype were analyzed at day 8 and day 16 in comparison with naive littermates. Data shown are from representative mice.

(B) Splenic cells from naive and immunized RAG2$^{+/+}$ and RAG2$^{GFP/GFP}$ mice were stained with PE-anti-B220 and bi-493, PE-anti-B220 and bi-GL-7, or PE-anti-IgM and bi-GL-7 and analyzed by FACS (bi-antibodies revealed with SA-Red613). A representative FACS plot is shown for analysis of splenic cells from naive, day 8, and day 16 mice for each set of markers, as there were no significant differences in phenotype between RAG2$^{+/+}$ and RAG2$^{GFP/GFP}$ naive or immunized spleens. For each of the gated populations from day 16 spleen (depicted by schematics), histograms for green fluorescence were generated. WT day 16 histograms (shaded) were overlayed with RAG2:GFP day 16 histograms (open); percentages of cells out of total live lymphocytes are shown for each population (top, WT; bottom, RAG2:GFP).

(C) Splenic cells from day 16 RAG2$^{+/+}$ mice were triple stained with PE-anti-B220, bi-493, and FITC-GL-7 or with FITC-anti-B220, bi-493, and PE-anti-IgM and analyzed by FACS. The population of pB130-140^{hi}B220^{lo} cells was gated for each sample and reanalyzed for the expression of GL-7 and IgM. The percentages of GL-7^{bright} versus GL-7^{dull} and IgM$^{-/lo}$ versus IgM^{+} cells in the gated pB130-140^{hi}B220^{lo} population are indicated.

pB130–140^{+} cells, approximately one-fourth of the cells are GL-7^{+} and three-fourths are GL-7^{-} (Figure 6C). RAG2:GFP expression is found in both the GL-7^{+} and GL-7^{-} subsets (Figure 6B). Thus, we have identified a novel population of RAG-expressing cells that appears in the spleen during the late stages of an immune response.

The RAG2:GFP^{+} splenic B cells that appeared at day 16 following immunization constituted a remarkable fraction of total B220^{+} cells, ranging from 2%–20%, depending on the mouse. Such a large population of phenotypically immature B cells in the spleen following immunization was unexpected. Furthermore, these cells clearly express the RAG2:GFP marker, implying that a much larger number of RAG-expressing cells are present in the spleen during the late stages of an immune response than was previously appreciated.

Increased RAG2:GFP Levels following BCR Cross-Linking of In Vitro–Generated sIgM^{+}sIgD^{+} BM Cells

Given our finding of large numbers of RAG2:GFP^{+} splenic B cells following immunization and recent reports of *RAG* reinduction in peripheral sIg^{+} cells, we sought to test whether RAG2:GFP expression could be reinduced in sIg^{+}, RAG2:GFP^{-} cells. First, we assayed RAG2$^{GFP/GFP}$ spleen cells treated with LPS and IL-4, but

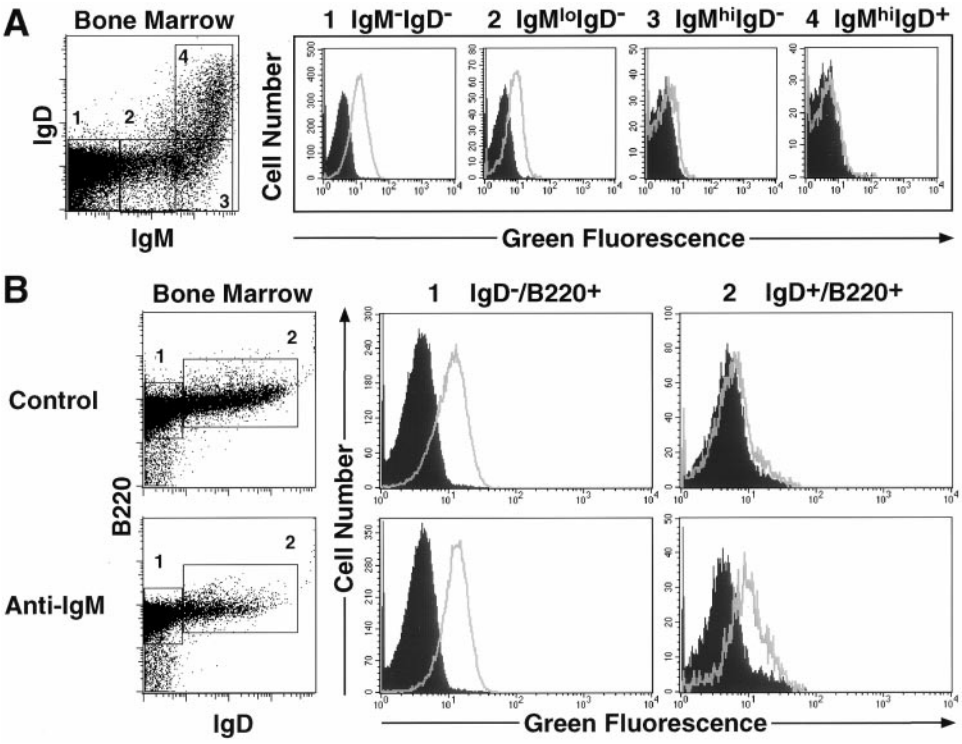

Figure 7. RAG2:GFP Expression in Cultured sIgD$^+$ B Cells

(A) BM isolated from RAG2$^{GFP/GFP}$ and RAG2$^{+/+}$ mice was plated on the T220 stromal line for 1–2 weeks. Cells were stained with PE-anti-IgM and bi-anti-IgD (revealed by SA-CyC) and analyzed by FACS. Histograms for green fluorescence were generated for gated populations 1–4; WT histograms (shaded) were overlayed with RAG2:GFP histograms (open).

(B) RAG2$^{GFP/GFP}$ and RAG2$^{+/+}$ BM cells were removed from the T220 stromal line and cultured for 48 hr in medium alone (control) or medium with 2 μg/ml F(ab′)$_2$ anti-IgM. Cells were then stained with PE-anti-IgD and CyC-anti-B220 and analyzed by FACS. Histograms for green fluorescence were generated for gated IgD$^-$B220$^+$ (1) and IgD$^+$B220$^+$ (2) populations. WT histograms (shaded) were overlayed with RAG2:GFP histograms (open).

the large blasting nature of these stimulated cells obviated fluorescent marker detection (data not shown). As another assay, we treated splenic RAG2$^{GFP/GFP}$ B cells with anti-IgM. We found no clear-cut increase in RAG2:GFP expression (data not shown), although we could have missed induction if it occurred in a small subpopulation of cells.

Based on previous studies (Melamed et al., 1998), we also assayed RAG2$^{GFP/GFP}$ B cells generated in vitro via culture of BM progenitors on the T220 stromal line. In these BM cultures, as in freshly isolated BM, RAG2:GFP expression was detectable in pro–B, pre–B, and immature B cell subsets but not in sIgM^{hi}sIgD$^+$ cells (Figure 7A). Addition of anti-IgM (2 μg/ml for 48 hr) to the cultures led to sIgM downregulation, such that we could no longer distinguish pre–B from previously sIgM$^+$ immature B cells. However, following treatment (in the presence or absence of T220 cells), the majority of the sIg$^-$ cells continued to express RAG2:GFP, and surviving sIgD$^+$ cells expressed significantly increased levels of RAG2:GFP compared to those of unstimulated sIgD$^+$ cells (Figure 7B). As the anti-IgM treatment also resulted in a significant loss of B220^{hi}/sIgD^{hi} cells, we considered the possibility that the apparent induction actually resulted from cellular selection. However, labeling studies with the fluorescent marker CFSE showed little or no division of sIgD$^+$ cells during the course of this analysis (K. S., preliminary data), and most cells were in the G1 phase of the cell cycle both in the presence or absence of BCR ligation (data not shown).

Discussion

A Model System for Studying *RAG* Expression during B and T Cell Development

To assess definitively *RAG* gene expression in individual cells and cell populations in primary and peripheral lymphoid tissues of mice, we developed a knockin strategy to introduce a *RAG2:GFP* fusion gene into the endogenous *RAG2* locus. Our goal was to generate a *GFP* reporter gene that accurately reflected endogenous *RAG2* locus expression. Therefore, the targeting strategy placed the *RAG2:GFP* gene under the control of endogenous *RAG2* regulatory elements and generated a fusion protein that preserved RAG2 degradation signals. As discussed below, RAG2:GFP developmental expression patterns precisely mimic those of endogenous RAG2; in addition, the RAG:GFP fusion protein mediates V(D)J recombination and normal lymphocyte development in a manner indistinguishable from the authentic RAG2 protein.

RAG2:GFP expression first appears at the early B and T cell stages in which *RAG* expression has been described (Rodewald and Fehling, 1998; Melchers and Rolink, 1999). In T cells, RAG2:GFP expression is downregulated following TCRβ, γ, and δ gene rearrangement and

β selection, upregulated at the onset of TCRα gene rearrangement in DP thymocytes, and finally downregulated again in SP thymocytes. In B cells, RAG2:GFP expression is downregulated in pro–B cells following IgH chain assembly and expression, upregulated in pre–B cells coincident with the onset of IgL chain gene rearrangement, maintained at reduced levels in many immature sIgM$^+$ B cells, and finally downregulated in sIgM^{hi} transitional B cells (see below). In both B and T cells, RAG2:GFP expression is tightly regulated between these developmental transitions (e.g., between DP and SP thymocyte stages). Furthermore, RAG2:GFP fusion protein levels, like those of wild-type RAG2, are downregulated between small resting and large cycling cells of a given population.

We conclude that the RAG2:GFP marker is a faithful indicator of *RAG2* expression. Therefore, the RAG2:GFP knockin mice allow *RAG2* expression to be assayed in lymphocyte populations through simple FACS analyses and electronic gating and obviate more cumbersome and often less informative approaches such as extensive cell sorting coupled with RNA and protein analyses. Moreover, this novel model permits more quantitative judgments with respect to cell numbers and relative expression levels in given populations than are possible through traditional sorting strategies.

RAG2:GFP Mice Allow Definition of a Population of *RAG*-Expressing Splenic B Cells

We have identified a population of RAG2:GFP–expressing splenic B cells in young mice that closely resembles the pre–B and immature B cell populations seen in BM. By sorting these cells and equivalent populations from wild-type mice, we confirmed that RAG2:GFP expression correlates with both *RAG1* and *RAG2* RNA expression. This population of *RAG*-expressing cells, representing approximately 2%–3% of total splenic lymphocytes at 4 weeks of age, decreases steadily in size through 8–10 weeks of age, at which point it is no longer detectable by FACS. As the spleen is a site of significant hematopoeisis around birth and as pre–B cells can be cloned from the spleen through 4 weeks of age (Rolink et al., 1993), many of these *RAG*-expressing splenic B cells in young mice could have originated from residual splenic B cell lymphopoeisis.

Some splenic *RAG*-expressing cells may also be derived from the BM, which becomes the major site of B cell development after birth. As the numbers of small pre-B-II and immature B cells in the BM peak at 4–5 weeks (Melchers and Rolink, 1999), it is possible that some of these cells prematurely leave the BM and take up residence in the spleen before it has been completely filled with more mature B and T cells. Previous studies in our laboratory have established a precedent for the transit of *RAG*-expressing pre-B-like cells from the BM to the spleen (Young et al., 1997; Shaw et al., 1999). The finding of significant numbers of splenic pre–B cells implies a potential role during normal B cell development. For example, it is possible that the continued presence of pre–B and immature B cells in the spleen might permit receptor editing in these cells that could eliminate B cell receptors specific for peripheral self-antigens.

Induction of Splenic RAG2:GFP$^+$ B Cells by Immunization

Our finding of a large number of RAG2:GFP$^+$ cells in spleens of immunized mice during the late stages of an immune response confirms and extends previous observations of *RAG* expression in GC B cells. We have shown that the GFP$^+$ cells are uniformly IgM$^-$ or IgM^{lo}, B220^{lo}, and pB130–140^{hi} and thus phenotypically very similar to the GFP$^+$ B cells in the spleens of young mice. As previously suggested, this population of *RAG*-expressing cells arising following immunization might be derived from more mature splenic B cells that have downregulated expression of sIgM and B220, reaquired expression of immature markers such as pB130–140, and reexpressed surrogate L chain and *RAG* genes (Han et al., 1997a). Alternatively, the splenic GFP$^+$ cells observed following immunization might represent pre–B/immature B cells generated within residual hematopoietic islands in the spleen or that prematurely exit the BM, as we have suggested for the GFP$^+$ splenic B lineage cells found in young RAG2:GFP mice. Potentially, our ability to subdivide these *RAG*-expressing, splenic pre-B-like cells into two populations based on expression of the pre–B/GC marker GL-7 may reflect differences in origin and/or function.

Our current studies do not distinguish between the various models for the generation of *RAG*-expressing splenic B cells following immunization. The model that *RAG*-expressing cells are derived from mature splenic B cells implies a remarkable reversal of B cell development in the germinal center (Han et al., 1996). However, the model that BM-derived pre–B cells potentially can be recruited in large numbers to join an immune response in the spleen would also represent a completely undescribed phenomenon in B cell development. In this regard, injection of certain exogenous agents into mice, such as sheep red blood cells, mineral oil, or BSA, can increase production of BM B cells (Fülop and Osmond, 1983), although it is not known whether immunization with various antigen/adjuvant preparations such as NP-CGG/alum leads to a similar upregulation of BM B cell production. The more interesting possibility is that the recruitment of pre–B/immature B cells to the spleen evolved to contribute to some aspect of the immune response, such as permitting positive selection of pre–B cells in the germinal center via the pre–B cell receptor.

RAG2:GFP Expression Decreases with Increasing Density of sIgM and Is Not Detectable in the Transitional B Cell Compartment in the Spleen

We have shown that RAG2:GFP expression in sIgM$^+$ B220^{lo} immature B cells in the BM correlates inversely with increasing sIgM density and is very low or absent in the IgM^{hi}B220^{lo} subset. In the spleen, we have demonstrated through FACS analysis and RT-PCR that *RAG* expression is very low or absent in sIgM^{lo}B220^{lo} cells and essentially absent in sIgM^{hi}B220^{lo} cells. This difference in *RAG* expression between phenotypically similar cells in the BM and the spleen strongly suggests that *RAG* expression is downregulated in these cells as they leave the BM. This downregulation may be due to the loss of a tonic signal provided by the BM microenvironment (Sandel and Monroe, 1999).

From a pool of 2×10^7 immature BM B cells generated each day, only 10%–20% get selected into the long-lived peripheral B cell pool (Allman et al., 1992). The site where most developing B cells are lost is not clearly established (Pillai, 1999). However, several studies suggested that transitional B cells, rather than immature B cells, are the major target of negative selection (Carsetti et al., 1995; Melamed et al., 1998). These studies have led to a set of related models that predict that antigen receptor signaling leads to distinct outcomes in immature versus transitional B cells. In response to self-antigen, immature B cells are proposed to undergo receptor editing, while transitional B cells are predicted to undergo apoptosis. Our finding that RAG:GFP expression is present in immature BM B cells but undetectable in transitional B cells in the spleen is consistent with these models.

Can *RAG* Genes Be Reexpressed in Peripheral B Cells?

Our analyses of the RAG2:GFP mice have led us to conclude that the vast majority of immature B cells downregulate *RAG* expression as they leave the bone marrow and enter the splenic transitional B cell compartment. However, work of others raised the general possiblity that *RAG* genes could be reexpressed in peripheral B cells (Han et al., 1997a; Papavasiliou et al., 1997; Hikida et al., 1998) and, more specifically, that a subset of transitional B cells retains the capacity to upregulate *RAG* genes when stimulated via their BCR in the presence of BM (Sandel and Monroe, 1999). In this regard, we found that anti-IgM treatment of in vitro–differentiated BM B cell cultures led to the appearance of IgD^+ cells that expressed RAG2:GFP. While the precise mechanisms of this apparent induction remain to be determined, our current results validate the use of the RAG2:GFP system to assess capacity of in vivo B lineage populations to modulate *RAG* gene expression in response to various stimuli.

Experimental Procedures

Construction of the *RAG2:GFP* Fusion Gene and Targeting Vector

A genomic clone containing the murine *RAG2* coding exon and several kilobases of 5′ and 3′ sequences was obtained. The *RAG2:GFP* fusion gene was generated by inserting a 2.2 kb SalI–AseI fragment containing the *RAG2* coding exon into the SacI site of the Clontech p*EGFP*-N1 vector (p*RAG2:GFP* #1). The junction was sequenced to confirm that the *RAG2:GFP* fusion was in-frame. p*RAG2:GFP* #1 was used in V(D)J recombination assays (data not shown) as described (Frank et al., 1998). A 750 bp AseI–KpnI fragment containing the ~330 bp 3′ UTR of *RAG2* was cloned into the NotI site of p*RAG2:GFP* #1 located just 3′ of the *RAG2:GFP* fusion gene to generate p*RAG2:GFP* #2. A 3.0 kb ClaI–SalI fragment containing the genomic region 5′ of the *RAG2* coding exon was cloned into the BglII site of p*RAG2:GFP* #2 to generate p*RAG2:GFP* #3. The *RAG2:GFP* knockin construct was generated by cloning a 5.8 kb XbaI fragment (containing the *RAG2:GFP* fusion gene and 2.2 kb of 5′ sequences) from p*RAG2:GFP* #3 and a 2.4 kb KpnI–NotI fragment (containing sequences 3′ of *RAG2*) into unique cloning sites in the pLNTK vector (Gorman et al., 1996).

Generation of RAG2:GFP Knockin ES Cells and Mice

TC1 ES cells were electroporated with 30 μg of PvuI-linearized *RAG2:GFP* knockin construct DNA and selected in media containing G418 and gancyclovir as described (Gorman et al., 1996). Targeted ES clones in which the *RAG2:GFP*(PGK-*neo*′) fusion gene had replaced the endogenous *RAG2* coding exon on one allele [$RAG2^{+/GFP(Neo+)}$] were identified by Southern blotting using the 5′ probe (1.0 kb ClaI–XbaI fragment) on EcoRI-digested DNA and confirmed using the 3′ probe (330 bp BglII–BamHI fragment) on EcoRI-digested DNA. $RAG2^{+/GFP(Neo+)}$ ES cells were transiently transfected with 30 μg of pMC-CreN; clones were isolated and screened by Southern blotting (EcoRI digest/3′ probe) for deletion of the PGK-*neo*′ gene. *RAG2:GFP*–targeted and PGK-*neo*′-deleted TC1 ES cells ($RAG2^{+/GFP}$) were used to generate chimeric mice by $RAG2^{-/-}$ blastocyst complementation as described (Chen et al., 1993). Male chimeras were bred to 129SvEv females to maintain a pure genetic background. Heterozygous $RAG2^{+/GFP}$ offspring were bred to generate $RAG2^{+/+}$, $RAG2^{+/GFP}$, and $RAG2^{GFP/GFP}$ mice. Genomic DNA isolation and Southern blotting were carried out as described (Gorman et al., 1996).

Flow Cytometry

Single-cell suspensions were stained with FITC-, PE-, CyC- and biotin-conjugated (bi) antibodies and analyzed by a FACSCalibur (Becton-Dickinson). The following antibodies were used (PharMingen and Southern Biotechnology Associates): FITC-anti-B220 (RA3-6B2); PE-anti-CD24 (M1/69), -CD25 (PC61), -CD4 (RM4-5), -TCRγδ (GL3), -CD43 (S7), -IgM^a (Igh-6a), -c-kit (2B8), -B220 (RA3-6B2), -IgD (11-26); CyC-anti-CD44 (IM7), -CD4 (RM4-5), -CD8 (53-6.7), -B220 (RA3-6B2); bi-anti-CD4 (RM4-5), -CD8 (53-6.7), -B220 (RA3-6B2), -MAC-1 (M1/70), -GR-1 (RB6-8C5), -IgD^a (Igh-5a/Igh-5.4). FITC- and bi-GL-7 (prepared by authors) and bi-493 (provided by M. Carroll) were also used. SA-PE, -CyC, -APC, and -Red613 were used to reveal bi-antibodies. For most FACS plots, ≥100,000 events were collected; dead cells were excluded by size and forward scatter gating. Data was analyzed with CellQuest (Becton Dickinson) or FlowJo (Tree Star) software. Cell sorting was performed on a MoFlo machine (Cytomation).

RT-PCR Analysis

Total RNA from sorted splenic populations was isolated using TRIzol (GIBCO-BRL). RNA samples were treated with DNase I AG (GIBCO-BRL), and reverse transcription of 0.5 μg of RNA was performed with SUPERSCRIPT II according to the directions of the manufacturer (GIBCO-BRL). PCR reactions (25 μl) contained 2% (or serial 5-fold dilutions) of the cDNA preparations, 3 pmol of both sense and antisense oligonucleotide primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 1 U Taq DNA polymerase in 1× PCR buffer (QIAGEN). Intron spanning primers for *RAG1*, *RAG2*, λ*5*, and β-*actin* were as described (Li et al., 1993) except that the λ5 sense primer was 5′-CTTGAGGGTCAATGAAGCTCAGA-3′. Amplification was performed on a GeneAmp 9600 thermocycler (Perkin-Elmer) employing the following conditions: 3 min at 94°C; 27 (β-actin) or 35 cycles of 45 s at 94°C, 90 s at 60°C, and 150 s at 72°C; and 10 min at 72°C. PCR products were resolved on 2% agarose gels, transferred to Zeta-Probe GT membranes (BioRad), and probed with ^{32}P-labeled cDNA for λ*5* or with cloned PCR fragments for *RAG1*, *RAG2*, and β-*actin*. Membranes were analyzed using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

Immunizations

$RAG2^{+/+}$ and $RAG2^{G/G}$ mice (10–12 weeks old) were immunized intraperitoneally with 100 μg of NP-CGG precipitated in alum as described (Han et al., 1997a). Spleens were taken at times indicated after immunization and dissociated into single-cell suspensions for FACS analysis.

BM Cultures

BM was plated on T220 stromal cells, an IL-7-secreting cell line, and cultured for a period of 1–2 weeks. For cross-linking experiments, cells were harvested and plated at ~1×10^6 cells/ml in medium ± 2 μg/ml $F(ab')_2$ anti-mouse IgM μ (Rockland) for 48 hr.

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