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Rearrangement of Mouse Immunoglobulin Kappa Deleting Element Recombining Sequence Promotes Immune Tolerance and Lambda B Cell Production

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SUMMARY

The recombining sequence (RS) of mouse and its human equivalent, the immunoglobulin (Ig) kappa deleting element (*IGKDE*), are sequences found at the 3′ end of the Ig kappa locus (*Igk*) that rearrange to inactivate *Igk* in developing B cells. RS recombination correlates with Ig lambda (Igλ) light (L) chain expression and likely plays a role in receptor editing by eliminating *Igk* genes encoding autoantibodies. A mouse strain was generated in which the recombination signal of RS was removed, blocking RS-mediated *Igk* inactivation. In RS mutant mice, receptor editing and self-tolerance were impaired, in some cases leading to autoantibody formation. Surprisingly, mutant mice also made fewer B cells expressing lambda chain, whereas λ versus κ isotype exclusion was only modestly affected. These results provide insight into the mechanism of L chain isotype exclusion and indicate that RS has a physiological role in promoting the formation of λ L chain-expressing B cells.

INTRODUCTION

The random nature of the B cell antigen receptor (BCR) gene assembly from variable (V), diversity (D), and joining (J) elements by the V(D)J recombinase often creates self-reactive specificities that are regulated by negative selection processes such as apoptosis or receptor editing [for review, see Nemazee (2006)]. In the process of receptor editing, B lymphocytes alter their antigen receptors through secondary immunoglobulin (Ig) gene rearrangements, usually involving the Ig kappa and lambda light (L) chain gene loci *Igk* and *Igl*. Editing often occurs in developing B cells that encounter autoantigens, resulting in the rescue of cells with reduced autoreactivity. The unique structure of the *Igk* locus, which allows secondary recombinations that can replace or destroy active *Igk* genes, facilitates receptor editing. Multiple recombinations can occur on a single *Igk* allele, owing to the presence of four functional Jκ elements, which can recombine to upstream Vκ elements (Feddersen and Van Ness, 1985; Shapiro and Weigert, 1987).

One DNA element predicted to have an exclusive role in receptor editing is the recombining sequence (RS) (Durdik et al., 1984), the mouse homolog of the human Ig kappa deleting element (*IGKDE*) (Siminovitch et al., 1987). RS lies 25 kb downstream of Cκ, carries a canonical Ig gene recombination signal, and rearranges by V(D)J recombination to Vκ elements and to sites in the Jκ-Cκ intron (reviewed by Selsing and Daitch, 1995). RS recombination results in deletion of the Cκ exon and silencing of the *Igk* locus. Almost all mouse and human λ B cells (75%–95%) and 10%–15% of κ B cells carry RS recombinations (Moore et al., 1985; Siminovitch et al., 1985; Nadel et al., 1990; Zou et al., 1993; Dunda and Corcos, 1997; Brauninger et al., 2001). Compared to mice, humans have a higher fraction of B cells that express λ-chain (40% versus 6%) or rearrange *IGKDE* (50% versus 20%) (Selsing and Daitch, 1995; Dunda and Corcos, 1997; Brauninger et al., 2001). In actively rearranging B cell lines, RS, *IGKDE*, and *Igl* recombinations are temporally correlated, but with rearrangements of RS and *IGKDE* preceding those of *Igl* (Persiani et al., 1987; Muller and Reth, 1988; Klein et al., 2005).

Because of the strong link between RS rearrangements and a cell's Igλ expression, Selsing and colleagues proposed that RS recombination might be required to trigger *Igl* locus recombination. RS rearrangement might "activate" RS or a locus downstream that would in turn promote *Igl* recombination (Persiani et al., 1987). Alternatively, RS rearrangement might eliminate a putative *cis*-acting suppressor of *Igl* recombination lying between RS and its recombination partner sites. Indeed, the RS element deletes both intronic and 3′ κ enhancers when rearranged (Muller et al., 1990). However, little evidence has been obtained to support these models. The RS element does not appear to encode any protein (Daitch et al., 1992), and the major homology between mouse and human RS elements is in their recombination recognition sites (Siminovitch et al., 1987). Immediately downstream of RS lies a housekeeping gene that is unlikely to play any B cell-specific role (Apel et al., 1995). Furthermore, mouse strains carrying different targeted deletions in the *Igk* locus have robust *Igl* recombination and B cell generation despite an impaired ability to recombine RS (Chen et al., 1993; Takeda et al., 1993; Zou et al., 1993). But it is not excluded that RS recombination, through excision of elements around Cκ, might promote *Igl* recombination by eliminating DNA elements competing for V(D)J recombinase (Daitch et al., 1992; Inlay et al., 2002).

We proposed an alternative hypothesis for RS function, namely that it plays a role in receptor editing (Tiegs et al., 1993; Retter and Nemazee, 1998) [a possibility also discussed by Selsing and Daitch (1995)]. RS recombination is elevated in artificial models in which B cells are arranged to be initially autoreactive (Chen et al., 1997; Pelanda et al., 1997; Aït-Azzouzene et al., 2005). RS and *IGKDE* recombinations often inactivate previously in-frame, functional *Igk* loci in normal individuals (Retter and Nemazee, 1998; Brauninger et al., 2001). In our study, 47% of such Vκ-Jκ remnant loci were in frame, suggesting that RS recombination was actively promoted by BCR signaling attributable to autoreactivity (Retter and Nemazee, 1998). We also found that RS recombinations usually occur when other options on the *Igk* locus run out, because 80% of the *Igk* loci in $sIg\lambda^+$ cells were inactivated by RS after rearranging to Jκ5, the last J element (Retter and Nemazee, 1998). We proposed that RS recombination likely reduces the frequency of cells with two different L chains, allowing receptor editing to be compatible with lymphocyte monospecificity (allelic and isotype exclusion). Increased RS recombination or λ B cell production associated with receptor editing were also described in autoantibody transgenic (Tg) models (Tiegs et al., 1993; Pelanda et al., 1997; Li et al., 2004). We recently studied Tg mice expressing on cell surfaces a synthetic superantigen reactive with Igκ. Bone marrow B cells in these so-called *κ-macroself* Tg mice undergo massive κ-to-λ editing characterized by increased RS recombination and λ B cell production (Aït-Azzouzene et al., 2005).

To investigate the role of RS in λ B cell production and receptor editing, we generated a mutant lacking the recombination signal sequence of RS. These mice manifest defects both in the ability to undergo receptor editing in response to autoantigen and in the generation of λ B cells. Importantly, these studies also reveal a role for receptor editing in the prevention of autoantibody formation.

RESULTS

Generating and Characterizing the $RS^{\Delta/\Delta}$ Mouse

RS mutant mice were generated with the scheme outlined in Figure S1 available online, in which the recombination signal of the RS element was removed and replaced with a neomycin resistance gene flanked by *loxP* sites. After cre-mediated deletion, the modified locus was verified to have the predicted sequence in which 139 bp encompassing the recombination signal of RS was substituted with a 199 bp stretch carrying a single *loxP* site and flanking vector sequences.

To evaluate functional effects of the RS mutation, we bred homozygous mutant ($RS^{\Delta/\Delta}$) mice and measured RS recombination to known recombination-signal sites in Vκ and the Jκ-Cκ intron by using polymerase chain reaction (PCR) assays of spleen and bone marrow (BM) cells. As shown in Figure 1, the RS mutation had the intended effect of blocking RS type recombination to Vκ (Vκ-RS) or to the major κ intronic site (RS to κ intron) (Figure 1A, top panels). By contrast, Vκ to Jκ recombination appeared to be normal (Figure 1A). We conclude that the germline RS mutation prevented normal RS rearrangements and had little effect on other recombinations at the *Igk* locus.

Because we discovered that λ B cell frequencies were reduced in $RS^{\Delta/\Delta}$ mice (see below), we also measured the extent of λ_1 excision product DNA formation in spleen and BM as an indicator of λ B cell production. PCR quantitation of λ_1 recombination excision circles in the BM $B220^+IgD^-$ cells revealed a 60%–70% reduction in $RS^{\Delta/\Delta}$ mice compared to the wild-type (Figure 1A lower panel, Figure 1B). Because DNA excision circles are not believed to replicate and are usually the result of nonfunctional recombination, these results indicate a reduced rate of *Igl* recombination in developing $RS^{\Delta/\Delta}$ B cells. Thus, the RS mutation correlated with a substantial, but incomplete, suppression of Vλ-Jλ recombination.

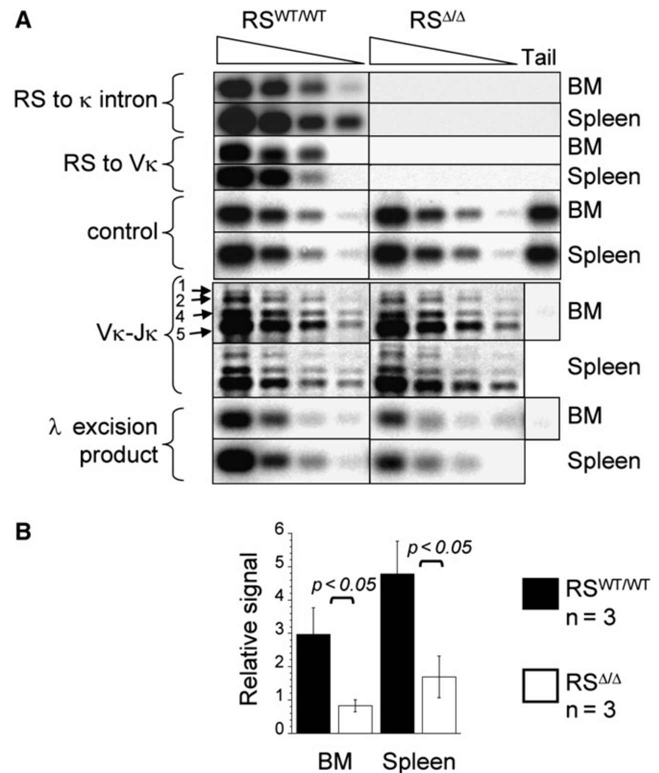

Figure 1. Evaluation of RS Recombination in Spleen and BM $B220^+$ Cells of $RS^{\Delta/\Delta}$ and Littermate $RS^{WT/WT}$ Mice

Four-fold serial dilutions of the indicated DNA samples were subjected to PCR reactions for the detection of the indicated DNA rearrangements. PCR products were electrophoresed on agarose gels and abundance quantitated by Southern blot with specific probes.

(A) Upper panels: PCR detection of RS recombination to JCκ intronic sites (RS to κ intron) and Vκ sites (RS to Vκ). Below are shown recombinations between Vκ and Jκ (Vκ-Jκ) and excision products of Vλ1 to Jλ1 recombination (λ excision product). Similar results were obtained in at least two additional independent experiments.

(B) Quantitation of $V\lambda_1$ to $J\lambda_1$ excision product in a sample of three mice/group. Shown are means with error bars indicating standard deviation (SD).

Reduction in the Frequency of λ B Cells in $RS^{\Delta/\Delta}$ Lymphocytes

Flow-cytometry analysis carried out on lymphoid tissues in a comparative sample of nine mice per group revealed that the frequency of $\lambda_{1\text{-}3}$ B cells in the spleens of $RS^{\Delta/\Delta}$ mice was reduced by approximately 42% compared to the wild-type (Figure 2A, right), which was mirrored in the decrease in absolute $\lambda_{1\text{-}3}$ B cell number (Table S1). Comparable reductions of $\lambda_{1\text{-}3}$ B

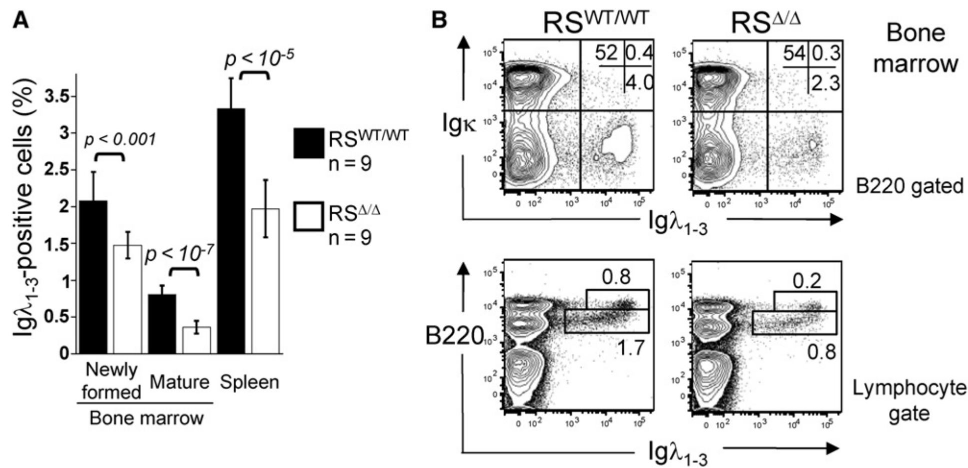

Figure 2. Evaluation of Reduced λ B Cell Frequencies in RS Mutant Mice

B cells from the BM and spleens of $RS^{\Delta/\Delta}$ and $RS^{WT/WT}$ mice were stained with antibodies to CD45R (B220), Igκ, and $Ig\lambda_{1-3}$ and analyzed by multicolor flow cytometry.

(A) Frequencies of cells carrying $Ig\lambda_{1-3}$ in the spleen and in newly formed ($B220^{intermediate}$) and mature ($B220^{high}$) BM B cells. A lymphocyte gate was used for the exclusion of bone-marrow myeloid cells.

(B) The upper plot shows costaining for κ and λ in $B220^+$ BM cells of the indicated mice. The lower panels show gating used in (A) to distinguish newly formed from recirculating sIg^+ B cells in BM.

Shown are mean values ± SD.

cells were seen in the BM, including among newly formed and recirculating cells (Figures 2A and 2B) and in all other tissues examined, including lymph nodes, peritoneal cavity, and blood (Figure S2). Igκ B cell numbers were normal or slightly reduced in analyses involving a total of 18 mice/group (Table S1). We conclude that RS mutation does not completely block λ B cell generation but lowers significantly λ B cell output from the BM ($p < 0.001$) and their steady-state numbers in the spleen ($p < 10^{-5}$).

Hybridoma Analysis

To further probe the effect of RS mutation, we generated a panel of B cell hybridomas and analyzed it for antibody L chain type and RS gene status (Table 1). Among 244 hybridomas generated from two wild-type mice, 22 (9%) secreted Igλ, whereas among hybridomas generated in five fusions of RS mutant mice, only 2.8% were λ^+ (17 of 603 hybridomas). Intracellular staining and flow-cytometry analysis of λ cells failed to detect cells coexpressing both κ and λ (data not shown). We conclude that, as sampled by hybridoma analysis, the frequency of λ expressing B cells is reduced in RS mutant mice and that cells expressing both κ and λ simultaneously are remarkably rare, regardless of RS mutation.

Because RS is usually recombined in λ-producing B cells, we tested λ-expressing hybridomas from mutant and wild-type mice for RS rearrangements by PCR as in Figure 1A and by Southern blotting with different restriction enzymes in

Table 1. Analysis of B Cell Hybridomas from $RS^{\Delta/\Delta}$ and $RS^{WT/WT}$ Mice for λ_{1-3} Secretion, κ λ_{1-3} Protein Coproduction, and RS Recombination

MouseGenotype and FusionNumber[a]	λ_{1-3} (%)	Number λ+ Hybridomas Found/Number Screened	λ+κ+	λ+κ−	Total λ+Frequency	RS Status of λ^+ Hybrids, Number Rearranged/Number Tested	Fraction of λ^+ Hybridomas with RS Recombination[b]
$RS^{WT/WT}$					9% (22/244)		100% (8/8)
1	7.4	2/27	0	2		1/1	
2	9	20/217	0	20		7/7	
$RS^{\Delta/\Delta}$					2.8% (17/603)		0% (0/9)
3	2.2	4/180	0	4		0/4	
4	5	4/80	0	4		0/3	
5	5	3/61	0	3		0/2	
6	1	1/92	0	1			
7	2.6	5/190	0	5			

[a] Each line represents a single fusion experiment with spleen cells from a different individual mouse. Hybridoma clones were screened for κ and λ_{1-3} immunoglobulin production as indicated in the Experimental Procedures.

[b] RS rearrangement status was assessed by PCR assay and genomic Southern blotting. Hybridomas were scored positive if at least one RS rearrangement was detected.

conjunction with an RS probe. Nine of nine tested $RS^{\Delta/\Delta}$ hybridomas lacked detectable RS rearrangements, whereas RS rearrangements were readily detected in wild-type λ-producing B cells (Table 1). These results supported the PCR assays of primary B cells (Figure 1A) in indicating that the mutant RS element does not detectably rearrange, even in B cells that eventually express λ chain. We conclude that although RS recombination does facilitate λ B cell development, substantial λ B cell development is possible in the apparent absence of RS element rearrangement.

Analysis of VκJκ5 Joins in λ B Cells

To test whether the reduction in λ cells in $RS^{\Delta/\Delta}$ mice was the result of counterselection of B cells with functional κ loci, we next cloned and sequenced VκJκ5 joins from sorted λ^+ B cells. [In wild-type mice, RS recombination often silences functional κ loci, usually leaving behind remnant Vκ joins involving the last Jκ element, Jκ5 (Retter and Nemazee, 1998).] We indeed found evidence of counterselection because only 10% (6/60) of VκJκ5 joins from $RS^{\Delta/\Delta}$ B cells were potentially productive compared to 34% (18/53) of wild-type joins. Such putative counterselection could occur before or after any actual λ recombination (see Discussion). In any case, this finding is consistent with the notion that λ B cells are often derived from κ cells carrying forbidden receptors that are silenced by RS-mediated editing.

Serum Antibody Analysis

$RS^{\Delta/\Delta}$ mice had comparable serum Ig titers to the wild-type, except that IgM,λ concentrations were reduced about 44% (10 μg/ml versus 17.9 μg/ml, $p = 0.0016$, $n = 9$). To see whether $RS^{\Delta/\Delta}$ mice had elevated amounts of spontaneous autoantibody, we measured by enzyme-linked immunosorbent assay (ELISA) IgM and IgG anti-dsDNA (dsDNA: double-stranded DNA) titers in sera of 7-month-old mice. Although not statistically significant, there was a trend to higher titers in the $RS^{\Delta/\Delta}$ group (Figure 3A). C57BL/6 mice normally do not develop DNA antibodies before 12 months of age (Morel et al., 1997). These tests for autoantibody were repeated with serum from a cohort of $RS^{\Delta/\Delta}$ mice carrying a B lineage-restricted *Bcl2* transgene (Strasser et al., 1990) and compared to results from $RS^{WT/WT}$;*Bcl2* Tg mice. In the context of enforced Bcl2 expression in B cells, the RS mutation significantly augmented anti-dsDNA levels over those present in $RS^{WT/WT}$;*Bcl2* Tg mice (Figure 3B; $p = 0.019$ IgG, $p = 0.004$ IgM).

Effects of RS Mutation on Receptor Editing

To measure the effect of RS mutation on central tolerance, we bred $RS^{\Delta/\Delta}$ mice to transgenic mice expressing ubiquitously a membrane-tethered Igκ superantigen (*κ-macroself* Tg mice) (Aït-Azzouzene et al., 2005) and assessed B cell numbers and phenotype by flow cytometry. In this context, all Igκ B cells are self-reactive. We previously showed that developing B cells in *κ-macroself* Tg mice undergo increased RS recombination and massive κ-to-λ editing. These mice essentially lack peripheral κ cells but have a modest increase in the steady-state frequency of BM κ cells. Most importantly, *κ-macroself* Tg mice have a 3-fold to 4-fold increase in the BM production of λ cells and a 7-fold increase in λ cells in the spleen (Aït-Azzouzene et al., 2005). RS mutant mice bred to the *κ-macroself* Tg background were found to have fewer λ cells in the spleen compared to RS-sufficient *κ-macroself* Tg controls (approximately 25% versus 35%, Figures 4A and 4D). The frequency of newly formed BM λ cells compared to RS-sufficient controls was similarly reduced in $RS^{\Delta/\Delta}$;*κ-macroself* Tg mice (Figures 4B and 4D). It appears that λ B cell development in the context of a Igκ-reactive superantigen is partly impaired in $RS^{\Delta/\Delta}$ mice.

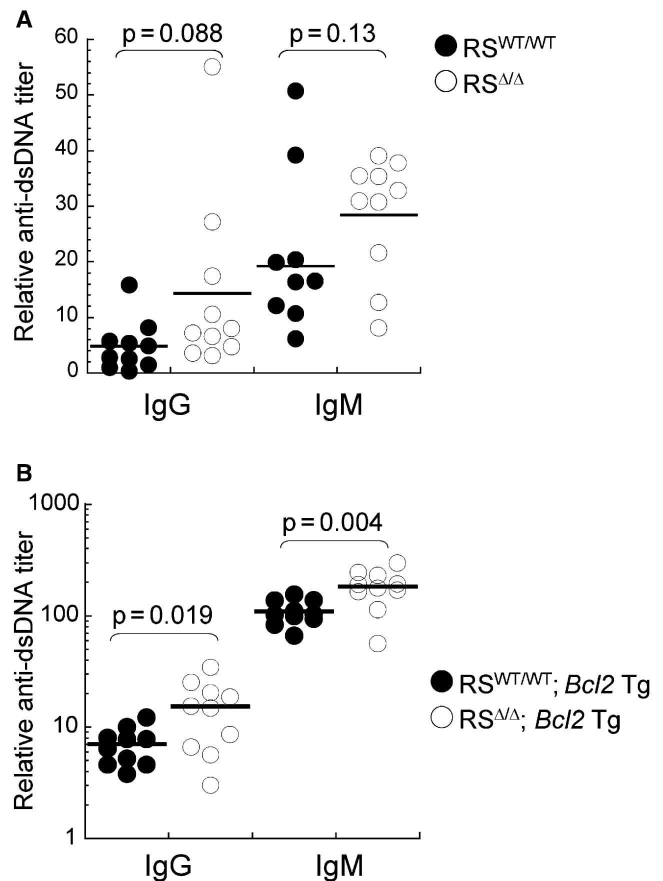

Figure 3. Analysis of dsDNA Antibody in Sera of the RS Mutant and Wild-Type Mice

Titers are relative to a reference high-titer serum pool from MRL/lpr mice that is given an arbitrary value of 100.

(A) Anti-dsDNA titers in 7-month-old $RS^{WT/WT}$ and $RS^{\Delta/\Delta}$ mice.

(B) Anti-dsDNA titers in 5-month-old $RS^{WT/WT}$;*Bcl2* Tg and $RS^{\Delta/\Delta}$;*Bcl2* Tg mice. Each dot represents the value obtained from a different individual mouse.

As expected, the κ-macroself antigen induced a loss of κ cells in the spleen in both $RS^{WT/WT}$ and $RS^{\Delta/\Delta}$ mice. But in RS mutants, there was a slight increase in the numbers of κ cells escaping to the periphery (Figures 4A and 4C). Moreover, in the BM of $RS^{\Delta/\Delta}$;*κ-macroself* Tg mice, the frequency of immature $B220^{intermediate}κ$ cells was significantly increased (Figures 4B and 4C). These additional, functionally autoreactive κ cells presumably represent those cells that were unable to silence *Igk* loci by RS recombination. We expected to also see a substantial population of cells coexpressing both κ and λ chains in BM of $RS^{\Delta/\Delta}$;*κ-macroself* Tg mice; however, the κλ double-expressing population was relatively small. We conclude that in the context of central negative selection of κ cells, the RS mutation leads to a significant ($p < 0.05$) reduction in λ cells and a slight increase in κ cells appearing in the peripheral immune system.

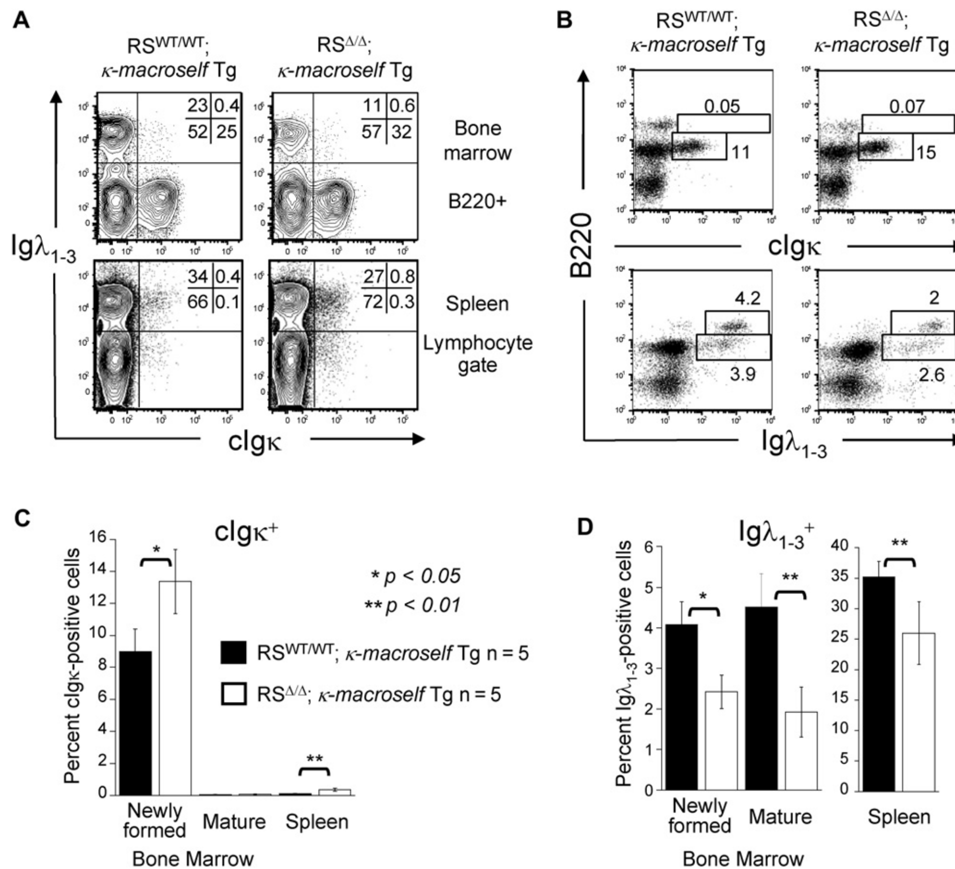

Figure 4. Analysis of IgL-Chain Expression of RS Mutant B Cells Developing in the Presence of a κ Superantigen
Spleen or BM cells of the indicated mice were simultaneously analyzed by multicolor flow cytometry for surface expression of Igλ and cytoplasmic expression of κ (cIgκ). Analyzed cells were gated on lymphocytes unless otherwise indicated. In (C) and (D), data shown are mean values ± SD.

Complementary Tolerance Mechanisms Revealed in $RS^{\Delta/\Delta}$ B Cells with Enforced Expression of Bcl2

In order to test the possibility that the reduction in λ B cell production in $RS^{\Delta/\Delta}$ mice was the result of the rapid death of κ cells carrying autoreactive receptors, we bred $RS^{\Delta/\Delta}$ mice to a B lineage-restricted *Bcl2* transgenic mouse (Strasser et al., 1990) and used their BM cells to reconstitute lethally irradiated mice carrying the *κ-macroself* Tg. The choice of a BM-transfer approach to challenge B cells with the superantigen (as opposed to introducing it by breeding) was one of convenience and also ensured that the superantigen was not expressed on B cells themselves. We reasoned that the κ-macroself antigen (Ag) would induce κ-to-λ editing and that enforced *Bcl2* expression would potentially rescue survival of κλ double-positive cells that might be generated in the absence of RS. The following differences were apparent in the recipients of $RS^{\Delta/\Delta}$;*Bcl2* Tg compared to *Bcl2* Tg cells lacking the RS mutation ($RS^{WT/WT}$;*Bcl2* Tg). First, the striking loss of BM κ^+ cells in the presence of κ-macroself Ag failed to occur in the recipients of $RS^{\Delta/\Delta}$;*Bcl2* Tg cells, indicating that much of the κ B cell loss in $RS^{WT/WT}$;*Bcl2* Tg mice was the result of RS-mediated editing rather than mere BCR downregulation (Figure 5A plots a and b, lower-right quadrants). Second, in $RS^{\Delta/\Delta}$ mice, fewer λ-positive cells were generated in the BM (Figure 5B, Figure 5A, compare plots a and b, upper-left quadrants) and spleen (Figure 5B, Figure 5A plots c and d). Third, the expected increase in κλ double-positive cells failed to occur. Finally, κ cells, which were not seen in the spleens of *κ-macroself* Tg recipients of $RS^{WT/WT}$*;Bcl2* Tg BM, were present in large numbers in spleens of mice that received $RS^{\Delta/\Delta}$*;Bcl2* Tg BM (Figure 5A plots c and d). Many of these κ splenocytes expressed markers of maturation, including CD21 and CD23, but they also expressed CD93 (Figure S3). These findings indicate that RS mutants have a defect in self-tolerance but a modest impairment in L chain isotype exclusion.

To determine whether the autoreactive B cells rescued in $RS^{\Delta/\Delta}$*;Bcl2* Tg→ *κ-macroself* Tg mice were functional, we assessed in these mice serum Igκ levels. As shown in Figure 5D, high serum titers of Igκ were found in IgM and IgG isotype fractions of animals receiving $RS^{\Delta/\Delta}$ but not $RS^{WT/WT}$ cells. We conclude that the *Bcl2* transgene can indeed promote survival and maturation of autoreactive B cells and that a defect in RS editing (the RS mutation) combined with a pure defect in B cell survival (*Bcl2* Tg) can lead to the escape of autoreactive B cells to the periphery. Moreover, because in recipients of $RS^{\Delta/\Delta}$*;Bcl2* Tg bone marrow κ^+ cells were rare in the spleen, these results indicate that RS-mediated editing efficiently "eliminated" autoreactive B cells despite their apoptotic defect.

DISCUSSION

RS and *IGKDE* have been thought to play roles in three key physiological processes: the regulation of *Igl* expression, L chain

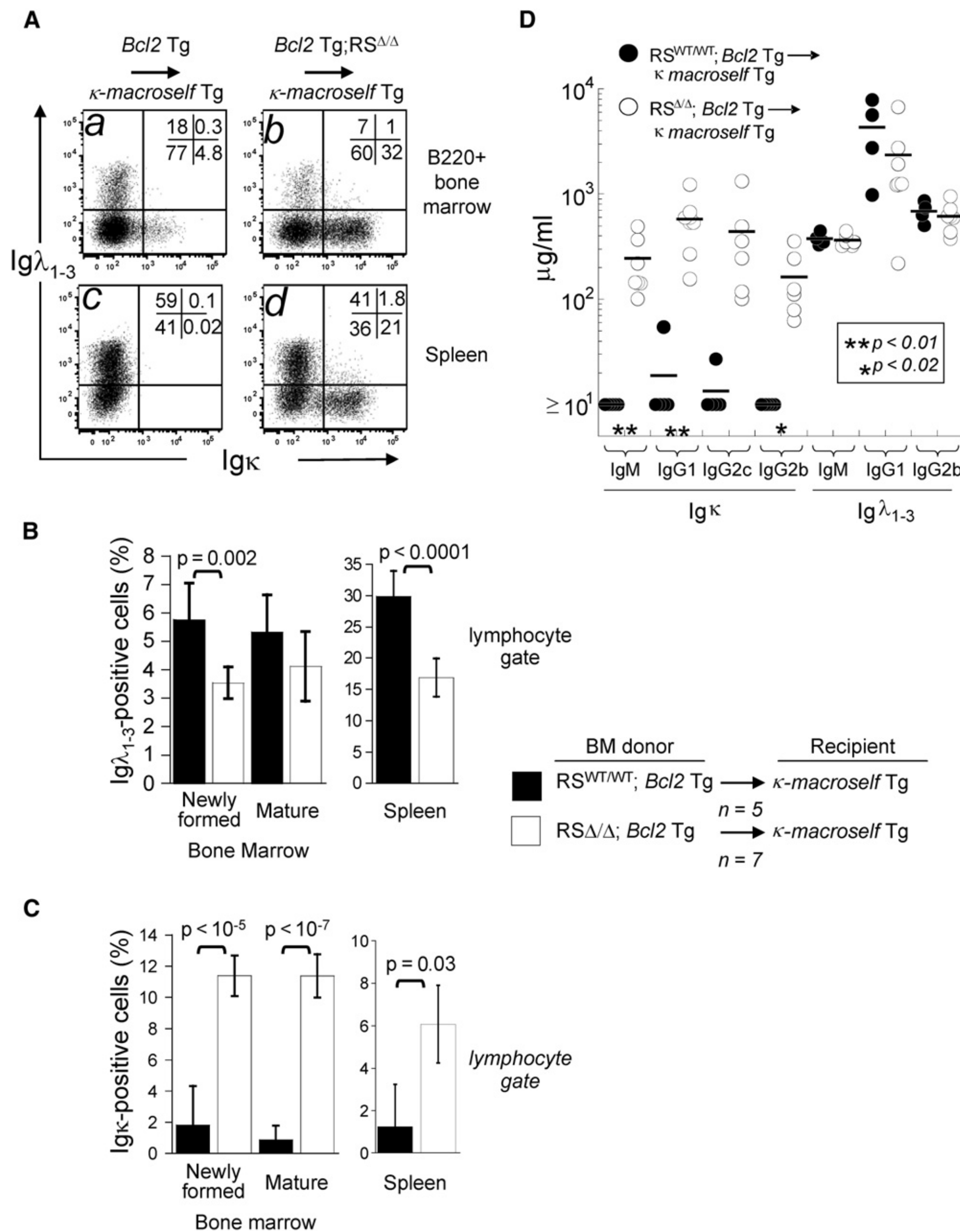

Figure 5. Analysis of the Effect of RS Mutation on B Cell Tolerance in Apoptosis-Resistant Cells

BM chimeras were generated with donor BM from RS-sufficient or RS mutant mice that also carried a *Bcl2* transgene enforcing B lineage-restricted expression. Irradiated recipient mice carried a ubiquitously expressed *κ-macroself* transgene (Aït-Azzouzene et al., 2005) to promote negative selection of κ^+ cells. Radiation chimeras were analyzed at 10 weeks after reconstitution.

(A) Analysis of λ and κ cell frequencies in BM and spleens of the indicated mice. (Note that κ staining in this figure involves surface staining rather than the cytoplasmic staining of permeabilized cells as shown in Figure 4A.)

(B and C) Summary analysis of the κ and λ frequencies of total splenic cells and newly formed ($B220^{intermediate}$) and recirculating bone marrow B cells ($B220^{high}$) from the indicated chimeras. Shown are mean values ± SD. Note increased κ frequency and reduced λ frequency among BM and spleen cells of *κ-macroself* recipients of $RS^{\Delta/\Delta}$;*Bcl2* Tg bone marrow.

(D) Igκ and Igλ serum immunoglobulin titers measured in the indicated chimeric mice.

isotype exclusion, and receptor editing. These effects are presumed to involve RS recombination itself, but until now, few studies have assessed RS function. An RNA transcript is associated with RS, but no protein-coding function is likely (Daitch et al., 1992). Our RS^{Δ} mutation blocked RS recombination, allowing evaluation of its putative functions. $RS^{\Delta/\Delta}$ mice had poor λ B cell production but surprisingly normal κλ isotype exclusion. Most importantly, $RS^{\Delta/\Delta}$ mice had a targeted defect in receptor editing that abrogated tolerance and promoted autoantibody formation, particularly in conjunction with an apoptotic defect contributed by *Bcl2* Tg expression.

In previous studies, mice with defective editing were engineered by rendering autoantibody Tg mice *Rag1* or *Rag2*

deficient; however, these mice manifest increased B cell deletion, rather than autoantibody formation (Spanopoulou et al., 1994; Xu et al., 1998; Halverson et al., 2004). Similarly, in conventional autoantibody Tg mice that coexpress the *Bcl2* Tg, central tolerance and editing are intact, and overt autoantibody formation is minimal (Hartley et al., 1993; Lang et al., 1997). Although *Bcl2* Tg overexpression by itself can suppress peripheral B cell tolerance, it does not hinder receptor editing (Lang et al., 1997; Aït-Azzouzene et al., 2005). We showed here that Bcl2 overexpression combined with the RS mutation led to frank autoimmunity, supporting the idea that central tolerance by receptor editing is complemented by apoptosis in cells that fail to edit expeditiously (Spanopoulou et al., 1994; Lang et al., 1997; Xu et al., 1998; Halverson et al., 2004).

The $RS^{\Delta/\Delta}$ mutation suppressed both λ B cell production and κ-to-λ receptor editing but affected κλ isotype exclusion only to a modest degree. B cells coexpressing both κ and λ are rare, but detectable (Zou et al., 1993; Pauza et al., 1993; Gollahon et al., 1988; Giachino et al., 1995; Diaw et al., 2000). The frequency of B cells with κ-chain allelic inclusion is estimated to be from 1.5% (Casellas et al., 2001) to 10% (Casellas et al., 2007). The latter study concludes that most cells scoring as included express only one κ-chain on the cell surface. We found that 10% (6/60) of λ splenic B cells in $RS^{\Delta/\Delta}$ mice carried a productive VκJκ5 rearrangement. The significance of the six apparently productive VκJκ joins is unclear. They might be defective in some way. In our hybridoma analysis, we failed to identify any κλ double-expressing hybrids from $RS^{\Delta/\Delta}$ or wild-type mice. In any case, the lack of a striking increase in κλ inclusion in $RS^{\Delta/\Delta}$ mice appears to demand the counterintuitive conclusion that the RS plays a minor role in enforcing κλ isotype exclusion, despite its critical role in κ-to-λ editing.

Our results contrast with studies involving targeted mutations within *Igk* that block both functional κ expression and RS recombination because those mutants have greatly increased, rather than decreased, *Igl* locus recombination and expression (Takeda et al., 1993; Chen et al., 1993; Zou et al., 1993; Xu et al., 1996; Inlay et al., 2002). Our $RS^{\Delta/\Delta}$ mutant permitted functional κ expression in the absence of RS recombination, revealing a specific but partial defect in *Igl* recombination and λ B cell production.

How does the $RS^{\Delta/\Delta}$ mutation suppress *Igl* expression? The receptor-editing view of RS function is that its purpose is to destroy *Igk* loci encoding autoreactive receptors, allowing progression to λ (Tiegs et al., 1993; Chen et al., 1997; Pelanda et al., 1997; Retter and Nemazee, 1998). One might suppose that without RS, autoreactive cells would be eliminated by clonal deletion or, failing deletion, would become allelically included. However, the dearth of $λ^+$ cells in RS mutants could not be attributed simply to the death of autoreactive κ B cells that failed to edit with RS. Even under artificial conditions in which apoptotic deletion of demonstrably autoreactive κ B cells was hindered by Bcl2 overexpression, λ B cell numbers were reduced and κλ double-positive cells were rare. Our results rule out premature apoptosis as an explanation for how λ B cell output is reduced in $RS^{\Delta/\Delta}$ mice. Rather, they support the previously abandoned idea that RS recombination promotes *Igl* rearrangement (Durdik et al., 1984). If RS recombination was needed to activate λ recombination directly, other mutants impaired in the ability to recombine RS should lack λ cells, which, as mentioned above, is not the case. Moreover, analysis of germline λ transcription in BM B cells of $RS^{\Delta/\Delta}$*;Bcl2* Tg→ *κ-macroself* Tg chimeras suggested that *Igl* locus accessibility was not reduced (Figure S4).

An alternative hypothesis to explain the linkage between recombination of RS and *Igl* is that when RS deletes Cκ and adjacent DNA, it removes *cis*-acting elements that compete for recombinational targeting with the *Igl* loci. This seems unlikely, however, because excision circles formed by RS recombination are retained in these nondividing cells and so should be free to provide competition for recombinase. Further, $RS^{WT/WT}$;*κ-macroself* Tg mice generate λ B cells as efficiently as do $JCκ^{\Delta/\Delta}$ mice (Aït-Azzouzene et al., 2005), though $JCκ^{\Delta/\Delta}$ mice lack intronic enhancer elements as well as any *Igk* locus or RS recombination (Chen et al., 1993). Third, in Cκ exon-targeted mice, in which Vκ-to-Jκ recombination but no RS recombination occurs, λ B cell production is comparable to that of $JCκ^{\Delta/\Delta}$ mice unable to recombine any κ loci (Zou et al., 1993; Chen et al., 1993). These considerations argue against a simple model of *Igk* versus *Igl* competition for recombinase.

The defect in *Igl* recombination in $RS^{\Delta/\Delta}$ mice appeared to occur selectively in cells expressing functional κ-chain. B cells expressing λ largely lacked any detectable κ expression and vice versa. Moreover, the 42% reduction of λ B cell production in $RS^{\Delta/\Delta}$ mice was similar to the proportion of cells predicted to normally extinguish in-frame κ genes by RS recombination (Retter and Nemazee, 1998) and was reflected in the amount of *Igl*-recombination excision circles. Consistent with this, VκJκ5 junctions in $λ^+$ B cells of $RS^{\Delta/\Delta}$ mice were rarely functional. That L chain isotype exclusion was robust in $RS^{\Delta/\Delta}$ mice appeared be the result of specific inhibition of *Igl* recombination in $sIgκ^+$ cells rather than counterselection of κλ cells after their formation.

An appealing alternative hypothesis to explain the dearth of λ B cell production in $RS^{\Delta/\Delta}$ mice is that induction of *Igl* recombination in cells undergoing receptor editing occurs most efficiently when κ protein expression has been silenced. According to this view, the reduced λ B cell production in $RS^{\Delta/\Delta}$ mice arises from their inability to silence κ protein expression in editing cells. This hypothesis was supported by the findings that relatively few λκ double-expressing cells were generated in the BMs of *κ-macroself*-antigen-expressing mice in which all κ cells were autoreactive and induced to carry out receptor editing, even when their survival was artificially enhanced by introduction of the *Bcl2* transgene. It appears that *Igl* gene activation occurs selectively in cells lacking sIg, and this happens after RS-mediated editing extinguishes κ expression. The relative inability of cells to undergo λ recombination when unable to silence expression of an autoreactive κ gene could occur if (1) immature autoreactive cells express less recombinase than do surface immunoglobulin (sIg)-negative (preB) cells, (2) recombinase amounts are limiting for editing, and (3) λ gene recombination requires more recombinase than does κ recombination. There is evidence to support all of these possibilities: In autoantibody Tg mice, bone marrow *Rag* messenger RNA (mRNA) expression, though high in the presence of autoantigen, was lower than in non-Tg controls dominated by small, sIg^- preB cells (Tiegs et al., 1993; Lang et al., 1996), and RAG expression in BM cells of $RS^{\Delta/\Delta}$*;Bcl2* Tg→ *κ-macroself* Tg chimeras was reduced compared to that of $RS^{WT/WT}$ controls (Figure S4). Second, heterozygous deficiency of *Rag1* suppresses markedly receptor editing

Table 2. Analysis of Recombination Signal Sequences of Mouse Antibody L Chain Loci Relative to Usage

	9-mer	7-mer	Nonconsensus Substitutions	Approximate Usage
Consensus	GGTTTTTGT	CACTGTG		
Jk1	---------	-------	0	33%
Jk2	a--------	-------	1	25%
Jk4	---------	-------	0	13%
Jk5	---------	-------	0	28%
Vk	---------	-------	0–2 (Average 0.8)	99%
RS	a----c--c	-------	3	20%
Vlambda1	t---c----	--t----	3	3%
Vlambda2	t---c----	--t----	3	2%
VlambdaX	a----c---	t------	3	1%
Jlambda1	-t------c	---a---	3	3%
Jlambda2	------g-g	--t----	3	3%
Jlambda3	-----ag-g	-------	3	<1%

Adapted from Ramsden and Wu (1991). Shown are the nonamer-heptamer elements of the signals and their deviations from the consensus at the top of the figure. The number of positions deviating from consensus were summed in the central column. The approximate usage of gene segments in total B cells of wild-type mice is shown at right. Data were derived from the following references: Ramsden and Wu (1991), Wood and Tonegawa (1983), Durdik et al. (1984), Eisen and Reilly (1985), Nadel et al. (1990), Shimizu et al. (1991), Luning Prak et al. (1994), and Dunda and Corcos (1997).

in vivo, indicating that *Rag1* is limiting for editing (Verkoczy et al., 2005; Aït-Azzouzene et al., 2005). Finally, *Igl* genes are known to be less efficiently recombined than are *Igk* genes because their recombination signals have more nonconsensus substitutions (Ramsden and Wu, 1991). Although the recombination signal of RS diverges from consensus to a similar extent as *Igl* elements, RS mainly joins with Vκ elements carrying consensus recombination signals (Table 2). RS recombination usually correlates with *Igl* recombination and proceeds after initiation of κ joining, but exceptions in which *Igl* rearrangement precedes *Igk* recombination have been identified both in normal and genetically modified individuals (Berg et al., 1990; Nadel et al., 1990; Chen et al., 1993; Zou et al., 1993; Dunda and Corcos, 1997). These exceptions might arise because *Igl* genes assemble best in developing B cells that lack κ protein, regardless of their RS recombination status. We therefore propose that in autoreactive B cells undergoing receptor editing, RAG protein expression is limiting and sufficient to drive Vκ, Jκ, and RS recombination but generally too low to drive λ recombination, which therefore occurs preferentially after destructive editing first renders the cell sIg negative.

EXPERIMENTAL PROCEDURES

Generation of RS Targeting Construct and Production of $RS^{\Delta/\Delta}$ Mice

Arms of homology flanking the RS recombination signal were cloned into the targeting vector pKO Scrambler NTV-1901flox (Stratagene). 129 strain bacterial artificial chromosome (BAC) clone DNA (230F18, Canadian Institutes of Health Research, Canada) was used as a template for high-fidelity PCR with Accuprime Pfx DNA polymerase (Invitrogen). Oligonucleotide primers were based on GenBank accessions numbers M12374 and AC090291. The following primers were used: long arm of homology, S1 5′-ACTAGTGTGTACCCCTC ACAGGTTGGTCCC-3′ SpeI and S3 5′-GCGGCCGGACTCATAAATCACAAC AAAGCAC-3′ NotI; and short arm of homology, S4 5′-CTCGAGGACCAGAGG GTTCAGTTCTTTGTC-3′ XhoI and S5 5′-ATCGATTCTCAGATTTGAGCCCTAA TGTAGC-3′ ClaI. After an initial 2 min incubation at 95°C, amplification conditions were 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, for 35 cycles. Restriction enzyme sites were included in the primer sequence for linearization of construct (NotI) and for genotyping of targeted locus (SpeI).

The targeting construct was linearized by digestion with NotI, isolated by agarose gel electrophoresis, and further purified with QIAEX II Gel Extraction Kit (QIAGEN). The knockout mice were produced by standard techniques at the Scripps Research Institute (TSRI) Mouse Genetics Core Facility. 129Sv/Ev embryonic stem (ES) cells were transfected by electroporation and selected in medium supplemented with G418. Clones carrying the appropriate targeted alleles were then identified by Southern blotting with appropriate restriction enzyme digestion along with independent probes derived from regions outside of the arms of homology.

Probe A was used both for the identification of homologous recombination and as a DNA PCR control for RS and excision product PCR assays (forward 5′-GGAAGTGCTCTAAGCAGTTGG-3′, reverse 5′-GGTAGGTGAGTGGTTCAG GAAGG-3′; 94°C 1 min, 58° C 1 min, 72° C 1 min, for 35 cycles). Probe B was used for confirmation of homologous recombination at the 5′ end (forward 5′-GAGGTTACTCAGCAAACCGTGG-3′, reverse 5′-TTCACACACGTGGCATA AACATACA-3′; 94° C 1 min, 58° C 1 min, 72° C 1 min, for 35 cycles).

Two targeted ES clones were selected for embryo aggregation, reimplantation, and selection for chimeric mice. An appropriately germline-targeted mouse containing the neomycin gene flanked by the two loxP sites was then backcrossed to the ZP3-Cre transgenic mouse (de Vries et al., 2000) for the removal of the neo cassette in vivo. RS-targeted mice were subsequently genotyped with the following primers: S1 Sequencing 5′-GGGACCAACCTGT GAGGGGTACAC-3′ and S5 Sequencing 5′-GCTACATTAGGGCTCAAATCT GAG-3′. PCR conditions were as follows: 94° C for 1 min, 60° C for 1 min, and 72° C for 1 min, for 29 cycles.

Mice

All mice were bred and maintained in the TSRI Animal Resources facility according to The Scripps Research Institute Institutional Animal Care and Use guidelines. C57BL/6J (B6) and B6.CD45.1 mice were from Jackson Laboratories. EmuBcl-2-22 transgenic (*Bcl2* Tg) mice (Strasser et al., 1991) were provided by A. Strasser and A. Harris (Walter and Eliza Hall Institute, Melbourne, Australia). *κ-macroself* Tg (line 2) was described (Aït-Azzouzene et al., 2005). $RS^{\Delta/\Delta}$ mice analyzed had been backcrossed ten times to B6 mice and were compared to B6 controls. In some experiments involving mice carrying *κ-macroself* and *Bcl2*, transgenes had been backcrossed six times to B6 or B6.CD45.1 mice. *Bcl2* Tg mice were initially on a mixed B10D2/B6 background.

Flow-Cytometry Analysis, Serum-Antibody Analysis, and Bone-Marrow Chimeras

Flow cytometry, serum-antibody analyses, and radiation bone-marrow chimera generation were essentially as described (Aït-Azzouzene et al., 2005).

Assay for dsDNA autoantibodies was carried out as follows: 10 μg/ml dsDNA from salmon sperm was coated to Nunc Maxisorp 96-well plates in 0.5× Reacti-Bind DNA coating solution (Pierce). After overnight coating, wells were blocked for 1 hr in Tris-buffered saline containing 5% nonfat dry milk powder. Mouse sera diluted in blocking solution supplemented with 1% bovine serum albumin (BSA) were applied and incubated for 90 min at 37°C. After extensive washing, bound antibodies were detected with 1:2000 diluted horseradish-peroxidase-conjugated goat anti-mouse IgM or goat anti-mouse IgG (Jackson Immunoresearch) and developed with 1-Step-Ultra TMB colorimetric substrate (Pierce). OD_{450nm} was measured with a Versamax plate reader (Molecular Devices). dsDNA-antibody concentration was normalized to a high-titer control serum kindly provided by D. Kono. Flow-cytometry data collection was done with an LSRII flow cytometer (Becton Dickenson) and analyzed with FlowJo software. For intracellular staining, surface-stained cells were fixed and permeabilized with a kit (Cytofix Cytoperm; BD Biosciences) and stained according to the manufacturer's instructions.

For BM transplantation, recipient mice carried the CD45.1 marker and in some cases carried the *κ-macroself* Tg. All BM donors were of the CD45.2 allotype. Ten weeks after reconstitution, recipients were sacrificed and their lymphoid tissues analyzed. Only chimeras in which at least 98% of cells in BM and spleen were donor derived were included in the analysis.

B Cell Isolation

BM cells were depleted of erythrocytes then incubated with an antibody cocktail including biotinylated antibodies to CD43, Ter119, CD4, IgD, and Gr-1. Cells with bound antibodies were removed by incubation with anti-biotin magnetic beads, and passage through LS columns (Miltenyi Biotec) followed. Unbound cells were collected. Splenic B cells were similarly isolated but without the use of IgD-depleting antibodies. The purity of these preparations was over 90% in all cases, as determined by B220 and CD19 staining.

PCR Assays for RS and Ig Gene Recombinations

PCR reactions were done in a final volume of 50 μl containing 50, 12.5, 3.1, or 0.78 ng of B cell or spleen cell genomic DNA. The Vλ1-to-Jλ1 excision product DNA rearrangements were done with the oligonucleotides and PCR conditions as described (Tiegs et al., 1993). RS-to-IκRS and RS-to-Vκ PCR assays were performed with primers B and C (Retter and Nemazee, 1998) along with a Vκ degenerate primer (Schlissel and Baltimore, 1989). Samples were amplified for 25–30 cycles of 1 min at 94°C, 1 min at 60° C, and 1 min at 72° C. PCR products were electrophoresed in 1.5% agarose gels, blotted on nylon membranes (Zeta-Probe membranes, Bio-Rad), and hybridized with radioactive probes as previously described (Aït-Azzouzene et al., 2005). Signals were quantified with a Phosphorimager with ImageQuant software (Molecular Dynamics).

Sequencing of VκJκ5 Joins

sIgλ^{+} splenocytes were magnetically sorted with anti-Ig$\lambda_{1\text{-}3}$ biotinylated antibody and anti-biotin microbeads (Miltenyi), and cell sorting (FACSAria, BD) followed. Bead-isolated cells were stained with anti-Igκ (187.1 Alexa 647), CD4 PerCP-Cy5.5, CD8 PerCP-Cy5.5, CD19 PE-Cy7, B220 PacificBlue, and streptavidin PE for the detection of the λ cells. Sorted $\lambda^{+}\kappa^{-}$ B cells were confirmed to be more than 98% Igλ^{+}. DNeasy kit was used for the isolation of genomic DNA from approximately 2,000,000 cells/sample. Vκ degenerate and Jκ5 primer (5′-TGCCACGTCAACTGATAATGAGCCCTCTCC-3′) were used for PCR amplification of VκJκ5 rearrangements. The PCR products were electrophoresed on 1% agarose gels, purified with a kit (QIAGEN), and cloned in PCR4-TOPO plasmid vector (Invitrogen), and the inserts were sequenced (Eton Bioscience). Sequence analysis was carried out with the Ig Blast program.

Hybridoma Generation and Analysis and Antibody Assays

B220^{+} spleen cells were cultured for 48 hr in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and lipopolysaccharide (LPS) (50 μg/ml) and fused with the SP2/0 myeloma line with polyethelene glycol. Cells from each fusion were then plated into 96-well plates and hybrids selected with hypoxanthine-aminopterin-thymidine (HAT) medium (ATCC). Because of the low plating density, no 96-well plate showed growth in more than 15 wells. Any wells with two distinct colonies were excluded. Cell supernatants were then screened for IgMκ or IgMλ and confirmed by intracellular staining for Igλ and Igκ. Enzyme-linked immunosorbent assays for antibodies and immunoglobulin levels in culture supernatants or mouse sera were carried out essentially as described (Gavin et al., 2006).

Statistical Analysis

Group comparisons were analyzed by 2-tailed Student's t test unless otherwise indicated. $p < 0.05$ was considered significant.

Supplemental Data

Four figures and three tables are available at http://www.immunity.com/cgi/content/full/28/2/161/DC1/.

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