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Recombination by the mouse immunoglobulin κ deleting element *RS* promotes immune tolerance and Igλ B cell production

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Summary

The recombining sequence (*RS*) of mouse and its human equivalent, the kappa deleting element (*kde*), are sequences found at the 3′ end of the immunoglobulin κ locus that rearrange to inactivate the κ-locus in developing B cells. *RS* recombination correlates with Igλ expression and plays a role in receptor editing by eliminating κ genes encoding autoantibodies. A mouse strain was generated in which the recombination signal of *RS* was removed in the germline, a model predicted to prevent specifically a fraction of receptor editing events. In *RS* mutant mice receptor editing and self-tolerance were impaired, in some cases leading to autoantibody formation. Surprisingly, *RS* mutant mice made fewer λ B cells, whereas λ/ κ isotype exclusion was only modestly affected. These results may provide insight into the mechanism of L-chain isotype exclusion and indicate that *RS* has a physiological role in λ B cell formation.

Keywords

receptor editing; recombining sequence; kappa deleting element; immune tolerance

Introduction

The quasi-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 machinery creates several quality control problems, including the production of cells carrying useless or dangerous specificities, and cells lacking any antigen receptor at all owing to out of frame rearrangements. Positive selection mechanisms, such as assembly of the preBCR and BCR complexes, promote survival and development of cells with antigen receptors and disfavor cells carrying out of frame rearrangements (Kitamura and Rajewsky, 1992; Spanopoulou et al., 1994; Young et al., 1994; Papavasiliou et al., 1995; Papavasiliou et al., 1996; Tze et al., 2005; Verkoczy et al., 2007). Negative selection by contrast reduces the frequency of cells carrying autoreactive receptors through the mechanisms of clonal elimination or receptor editing (Goodnow et al., 1989; Nemazee and Burki, 1989; Erikson et al., 1991; Russell et al., 1991; Cyster et al., 1994).

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In the process of receptor editing B lymphocytes alter their antigen receptors through secondary antibody gene rearrangements, usually involving the κ and λ light chain gene loci. The editing response often occurs in developing B cells that encounter autoantigens, resulting in the rescue of cells with reduced or abolished autoreactivity. Receptor editing is particularly facilitated by the unique structure of the *Igκ* locus, which promotes secondary recombinations that can replace or destroy active κ genes. Multiple sequential κ locus recombinations can occur on a single allele owing to the presence of four functional *J* elements which can recombine to upstream *V* elements (Feddersen and Van Ness, 1985; Shapiro and Weigert, 1987).

One DNA element that is predicted to have an exclusive role in receptor editing and the silencing of κ light chain genes is the Recombining Sequence (*RS*) (Durdik et al., 1984). *RS* is the mouse homologue of the immunoglobulin kappa deleting element (*κde*) of humans (Siminovitch et al., 1987). It lies ~25kb downstream of the *Cκ* exon, carries a canonical Ag receptor gene recombination signal with a two-turn spacer, and rearranges by V(D)J recombination to sites upstream, including to germline *Vκ* elements and to two sites in the *Jκ-Cκ* intron (IRS1 and IRS2) (Durdik et al., 1984; Moore et al., 1985; Siminovitch et al., 1985; Siminovitch et al., 1987; Klobeck and Zachau, 1986; Shimizu et al., 1991; Selsing and Daitch, 1995). *RS* recombination results in the physical deletion of *Cκ* and the silencing of the κ locus. This natural "κ knockout" process frequently occurs in the development of mammalian B cells. Almost all mouse and human Igλ B cells (75–95%), and a significant subset of Igκ B cells (10–15%), carry *RS* recombinations (Durdik et al., 1984; Moore et al., 1985; Siminovitch et al., 1985; Nadel et al., 1990; Zou et al., 1993; Dunda and Corcos, 1997; Brauninger et al., 2001). In actively rearranging transformed B cells, *RS/kde* and λ recombination are temporally and developmentally correlated, but with *RS* recombination preceding λ recombination (Persiani et al., 1987; Muller and Reth, 1988; Klein et al., 2005).

Because of the strong link between *RS* rearrangements and a cell's Ig the presence of expression, Selsing and colleagues proposed that *RS* recombination might be required to trigger λ locus recombination (Durdik et al., 1984; Moore et al., 1985; Siminovitch et al., 1987; Persiani et al., 1987; Daitch et al., 1992; Selsing and Daitch, 1995). *RS* rearrangement might "activate" *RS* (or a locus downstream of *RS*) that would in turn promote λ recombination (Persiani et al., 1987). Alternatively, *RS* rearrangement might eliminate a putative cis-acting suppressor of λ recombination lying between *RS* and its recombination partner sites. Such a suppressor might be associated with transcriptional enhancers themselves as the *RS* element is downstream of both intronic and 3′ κ enhancers (Muller et al., 1990). However, little evidence has been obtained to support these models: *RS* does not recombine in a fixed frame relative to its recombination partners *Vκ*, *IRS1* and *IRS2* (Daitch et al., 1992). Moreover, and notwithstanding that *RS* transcription occurs in potentially recombining cells, the *RS* element does not appear to encode any protein, and the major homology between mouse and human *RS* elements is in their eptamer/nonamer 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 knockouts in the *Igκ* locus appeared to have robust λ-locus recombination and B cell generation despite an impaired ability to recombine *RS* (Chen et al., 1993; Takeda et al., 1993; Zou et al., 1993).

However, it is not excluded that *RS* recombination, through deletion of enhancer elements adjacent to *Cκ*, might promote λ-locus recombination by eliminating potentially competing DNA elements (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 (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* recombinations often inactivate previously in-frame, functional *Igκ* loci even in normal mice (Retter and Nemazee, 1998) and humans (Brauninger et al., 2001). In our study, 47% of such *Vκ-Jκ* remnant loci were in-frame, arguing that *RS* recombination was actively promoted by BCR signaling, presumably because of autoreactivity (Retter and Nemazee, 1998). We also found that *RS* recombinations usually occur when other options on the *κ*-locus run out, as 80% of the *κ* loci in sIgλ^+ cells were inactivated by *RS* after rearranging the last *J* element, *Jκ5* (Retter and Nemazee, 1998). We proposed that the *RS* locus is specialized to destroy autoreactive *κ* genes, and to reduce the frequency of cells with two different L-chains, allowing receptor editing to be compatible with lymphocyte monospecificity ("allelic/haplotype exclusion"). Increased *RS* recombination and λ B cell production associated with receptor editing were also described in autoantibody transgenic models (Tiegs et al., 1993; Pelanda et al., 1997; Li et al., 2004). More recently, we investigated mice expressing a synthetic superantigen gene that drives ubiquitous expression of a cell surface protein reactive with immunoglobulin κ. Bone marrow B cells in mice carrying the so-called *κ-macroself* transgene undergo massive *κ*-to-*λ* editing characterized by increased *RS* recombination and increased 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 mouse mutant lacking the *RS* element's heptamer/nonamer recombination signal. These mice manifest defects both in the ability to undergo receptor editing in response to autoantigen and in the generation of λ B cells. These studies also reveal a role for receptor editing in preventing autoantibody formation.

Results

Generating the *RS−/−* mouse

Knockout mice were generated using the scheme outlined in Supplementary Fig 1 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.

Assessing the success of the knockout

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

As we discovered that λ B cell frequencies were reduced in *RS*−/− mice (see below) we also measured *λ* excision product DNA levels in spleen and BM as an indicator of λ B cell production. PCR quantitation of the generation of $Ig\lambda_1$ recombination excision circles in the BM B220$^+$/IgD$^-$ cells revealed a 60–70% reduction in *RS*−/− mice compared to wild type (Fig 1A lower panel, Fig 1B). Because DNA excision circles are not believed to replicate and are usually the result of non-functional recombination, these results indicate a reduced rate of *Igλ* recombination in developing *RS*−/− B cells. Hence *RS* mutation was correlated with a significant, but incomplete, suppression of *Vλ-Jλ* recombination.

Reduction in the frequency of λ B cells in *RS−/−* lymphocytes

Flow cytometry analysis carried out on lymphoid tissues in a comparative sample of 9 mice per group revealed that the frequency of λ_{1-3} B cells in the spleens of *RS*−/− mice was reduced by ~42% compared to wild type (Fig 2A, right), which was mirrored in the decrease in absolute λ_{1-3}B cell number (Supplementary Table 1). Similar significant reductions of λ_{1-3}B cells were seen in the BM, including among newly formed and recirculating cells (Fig 2A,B), and in all other tissues examined, including lymph nodes, peritoneal cavity and blood (Supplementary Fig 2). κ B cell numbers were normal or slightly reduced in analyses involving a total of 18 mice/group (Supplementary Table 1). These results support the conclusion that *RS* mutation does not completely block λ B cell generation, but lowers significantly λ B cell output from the BM and their steady state numbers in the spleen.

Hybridoma analysis

To further probe the effect of *RS* mutation, a panel of B cell hybridomas was generated and analyzed for antibody L chain type and *RS* gene status (Table 1). Among 244 hybridomas generated from 2 wild type mice, 22 (9%) secreted Igλ, whereas among hybridomas generated in 5 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.

As *RS* is usually recombined in λ-producing B cells, we tested λ-expressing hybridomas from mutant and wildtype mice for *RS* rearrangements by PCR as in Fig 1A, and by southern blotting using different restriction enzymes in conjunction with an *RS* probe. Nine of 9 tested *RS*−/− hybridomas lacked detectable *RS* rearrangements, whereas *RS* rearrangements were readily detected in wildtype λ producing B cells (Table 1). These results supported the PCR assays of primary B cells (Fig 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, significant λ B cell development is possible in the apparent absence of *RS* element rearrangement.

Analysis of *VκJκ5* joins in λ B cells

To test if the reduction in λ cells in *RS*−/− 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*−/− 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−/− mice had comparable serum immunoglobulin levels to wild type, except that IgM, λ levels were reduced about 44% (10 μg/ml vs 17.9 μg/ml, p=.0016, n=9). To see if *RS*−/− mice had elevated spontaneous autoantibody levels, we measured IgM and IgG anti-dsDNA levels in sera of seven month old mice by ELISA. not statistically significant, there was a trend to higher level binding in the *RS*−/− group (Fig 3A). C57BL/6 mice normally do not develop anti-DNA antibodies before 12 months of age (Morel et al., 1997). These tests for autoantibody were repeated using serum from a cohort of *RS* knockout mice carrying a B-lineage restricted

Bcl2 transgene (Strasser et al., 1990) and compared to results from *RS+/+;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+/+;Bcl2* mice (Fig 3B).

Effects of *RS* mutation on receptor editing

To measure the effect of *RS* mutation on central tolerance, we bred *RS−/ −* mice to transgenic mice expressing ubiquitously a membrane-tethered Igκ superantigen (κ-*macroself Tg* mice) (Aït-Azzouzene et al., 2005) and assessed B cells 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 3-4-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 *RS*-sufficient *κ-macroself Tg* controls (ca. 25% vs 35%, Fig 4 A,D). The frequency of newly formed BM λ cells compared to *RS*-sufficient controls was similarly reduced in *RS−/ −;κ-macroself Tg* mice (Fig 4 B,D). It appears that λ B cell development in the context of a Igκ-reactive superantigen is partly impaired in *RS−/ −* mice.

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

Complementary tolerance mechanisms revealed in *RS−/−* B cells with enforced expression of Bcl2

In order to test the possibility that the reduction in λ B cell production in *RS−/ −* mice was the result of the rapid death of κ cells carrying autoreactive receptors, we bred *RS* knockout mice to the B-lineage restricted *Bcl2* transgenic mouse (Strasser et al., 1990) and used their BM cells to reconstitute lethally irradiated mice carrying the κ-macroself Ag. The choice of a BM transfer approach to challenge B cells with the superantigen (as opposed to introducing the superantigen 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 Ag would induce *κ*-to-*λ* editing and that enforced *Bcl2* expression would potentially rescue survival of κ/ λ double positive cells that might be generated in the absence of *RS*. The following differences were apparent in the recipients of *RS−/ −;Bcl2Tg* compared to *RS*+/+;*Bcl2Tg* cells. First, the striking loss of BM κ^+ cells in the presence of κ-macroself Ag failed to occur in the recipients of *RS−/ −;Bcl2Tg* cells, indicating that much of the κ B cell loss in *RS*+/+;*Bcl2Tg* mice was the result of *RS*-mediated editing, rather than mere BCR downregulation (Fig 5A *a*,*b*, lower right quadrants). Second, in *RS−/ −* mice fewer λ positive cells were generated in the BM (Fig 5B, Fig 5A, compare *a*,*b*, upper left quadrants) and spleen (Fig 5B, Fig 5A plots *c*,*d*). Third, the expected increase in κ/ λ double positive cells failed to occur. Finally, κ cells, which were not seen in the spleens of *κ-macroself Tg* recipients of *RS+/+;Bcl2Tg* BM, were present in large numbers in spleens of mice that received *RS−/ −;Bcl2Tg* BM (Fig 5A*c*,*d*). Many of these κ splenocytes expressed markers of maturation, including CD21 and CD23, but they also expressed CD93

(Fig S3). These findings indicate that *RS* mutants have a defect in self-tolerance but a modest impairment in κ/ λ isotype exclusion.

To determine if the autoreactive B cells rescued in *RS−/ −;Bcl2 Tg→ κ-macroself Tg* mice were functional, we assessed in these mice serum κ immunoglobulin levels. As shown in Figure 5D, high serum levels of Igκ were found in IgM and IgG isotype fractions of animals receiving *RS−/ −*, but not *RS*+/+ 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 cell survival (*Bcl2Tg*) can lead to the escape of autoreactive B cells to the periphery. Moreover, because in recipients of *RS*+/+*;Bcl2Tg* BM κ^+ cells were rare in the spleen, these results indicate that *RS*-mediated editing efficiently "eliminated" autoreactive B cells despite their apoptotic defect.

Discussion

The *RS/κde* element has been suggested to play an evolutionarily conserved role in three important and possibly interrelated physiological processes: the regulation of *Igλ* gene expression, κ/ λ L-chain isotype exclusion, and receptor editing (Durdik et al., 1984; Siminovitch et al., 1985; Siminovitch et al., 1987; Muller and Reth, 1988; Muller et al., 1990). These effects were presumed to involve directly or indirectly *RS* recombination itself. However, until now few direct experiments have been carried out to specifically assess *RS* function. A specific RNA transcript is associated with the *RS* locus, which may play a role in recombinase accessibility, but no *RS* protein coding function is likely (Daitch et al., 1992). We show here that a targeted mutation of the *RS* recombination signal blocked *RS* recombination, allowing an assessment of its putative functions. *RS* mutant mice had defective λ B cell production, but surprisingly normal κ/ λ isotype exclusion. Most importantly, we have generated a mutant with a targeted defect in receptor editing and have demonstrated that this defect can abrogate tolerance, particularly in conjunction with an apoptotic defect contributed by enforced Bcl2 expression.

To our knowledge this is the first demonstration of a specific defect in receptor editing that promotes autoantibody formation. In other studies, mice with defective editing were engineered by rendering autoantibody transgenic mice RAG gene deficient, however, these mice manifested increased B cell deletion, rather than autoantibody formation (Spanopoulou et al., 1994; Xu et al., 1998; Halverson et al., 2004). Similarly, conventional autoantibody transgenic mice have been bred to a background expressing a B cell restricted *Bcl2* transgene (Hartley et al., 1993; Lang et al., 1997). In that context, central tolerance and editing appeared to be intact, and overt autoantibody formation was minimal. However, in mice in which B cells encountered antigen solely in the periphery, presumably at a developmental stage at which they were no longer competent to undergo receptor editing, self-tolerance was broken by Bcl2 overexpression (Lang et al., 1997). In the present study and earlier work we found that Bcl2 overexpression alone did not hinder receptor editing, and central tolerance proceeded efficiently (Lang et al., 1997; Aït-Azzouzene et al., 2005). However, we show here that Bcl2 overexpression combined with the *RS* mutation led to frank autoimmunity. Our findings reinforce the suggestion that central tolerance by receptor editing is normally complemented by developmental arrest and apoptosis in cells that fail to edit in an appropriate time frame (Spanopoulou et al., 1994; Lang et al., 1997; Xu et al., 1998; Halverson et al., 2004).

Compared to mice, humans have a much higher proportion of B cells that express Igλ L-chain (40% vs 6%) and that have rearranged the *RS/kde* element (50% vs 20%) (Durdik et al., 1984; Siminovitch et al., 1985; Dunda and Corcos, 1997; Brauninger et al., 2001). It therefore seems likely that the *kde* element plays an even more important role in humans than does the *RS* element of mice.

Lack of a functional recombination signal adjacent to *RS* suppressed significantly both λ B cell production and *κ*-to-*λ* receptor editing, but this mutation affected κ/ λ isotype exclusion only to a modest degree. It has been noted before that B cells expressing 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). However, it is interesting in this regard that, as in our study, few hybridomas could be isolated from normal mice that coexpress κ and λ (Gollahon et al., 1988). The frequency of B cells with κ-chain allelic inclusion has been estimated to be from 1.5% (Casellas et al., 2001) to 10% (Casellas et al., 2007). The latter study concluded that most cells that scored as included expressed only one chain on the cell surface. We found that 10% (6/60) of λ splenic B cells in *RS*−/ − mice carried an in-frame κ rearrangement. The significance of the remaining in-frame κ genes is unclear. Some may represent contamination of κ cells, others rearranged κ genes rendered non functional by defects outside of the region sequenced or cells carrying κ chains that are poorly expressed or unable to pair with the cell's H-chain. In our hybridoma analysis we failed to identify any κ/ λ protein double expressing hybrids from *RS*−/ − or wild type mice. In any case, our results indicating no striking increase in κ/ λ inclusion in *RS*−/ − mice appear to demand the counter-intuitive conclusion that the *RS* element plays a minor role in enforcing κ/ λ isotype exclusion, despite its critical role in promoting *κ*-to-*λ* editing.

It is of interest to contrast our conclusions with earlier studies involving targeted mutations of the *κ* locus. These include targetings that introduced neomycin genes within the *J-C* region of the *κ* locus (Takeda et al., 1993; Chen et al., 1993; Zou et al., 1993) and others that involved deletions of transcriptional regulatory elements with minimal introduction of heterologous sequence (Xu et al., 1996; Inlay et al., 2002). Many of these mutations blocked both functional κ expression and *RS* recombination owing to defects in cis-acting transcriptional regulators that affect recombinational targeting. These combined defects in κ expression and *RS* recombination were correlated with greatly increased, rather than decreased, *λ* locus recombination and expression, and appeared to exclude a required role for *RS* recombination in *Igλ* recombination and expression. Our present study permitted functional κ expression in the absence of *RS* recombination, revealing a specific, but partial defect in *Igλ* recombination and λ B cell production as a consequence of defective *RS* recombination.

How does *RS* mutation suppress *λ* gene expression? The receptor editing view of *RS* function is that its purpose is to destroy *κ* 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, they would become allelically included. Several studies indicate that failed editing of developing autoreactive B cells leads to deletion (Spanopoulou et al., 1994; Xu et al., 1998; Halverson et al., 2004). However, our data indicate that the dearth of λ cells in *RS* mutants cannot be attributed simply to the death of autoreactive κ B cells that fail to edit using *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 κ/ λ double positive cells were rare. Our results appear to rule out premature clonal deletion as an explanation for how λ B cell production is reduced in *RS*−/ − mice. Rather, the results support the previously abandoned suggestion that *RS* recombination is required to promote *Igλ* rearrangement (Durdik et al., 1984).

If *RS* recombination was needed to activate *λ* recombination directly, for example, through the activation of a trans-acting gene product encoded in the *κ* locus, mutants impaired in the ability to recombine *RS* should lack λ cells. However, several published experiments involving targeting of other elements in the *κ* locus have shown that λ B cells can be generated in the absence of *RS* recombination, often at enhanced efficiency (Chen et al., 1993; Takeda et al., 1993; Zou et al., 1993; Aït-Azzouzene et al., 2005). Moreover, analysis of germline *λ*

transcription in BM B cells of *RS−/ −;Bcl2 Tg→ κ-macroself Tg* chimeras suggests that *RS* mutation does not reduce accessibility of the λ locus (Fig S4).

An alternative hypothesis to explain the linkage between *RS* recombination and λ locus recombination invokes a putative enhancer competition for recombinational targeting. As *RS* recombination deletes the *Cκ* locus, it is predicted to change the context of the major cis acting elements adjacent to *Cκ*. In the absence of these competing cis acting elements, recombinational targeting to the λ loci might be more efficient. However, we do not favor this hypothesis for the following reasons. First, because excision circles formed by *RS* recombination are generated in non dividing cells, these often large episomes are not typically lost, so would in theory be available to provide competition in binding to any recombinational targeting machinery. Second, *κ-macroself Tg* mice on a *RS*-sufficient background generate λ B cells just as efficiently as do *JCκ* deleted mice (Aït-Azzouzene et al., 2005), though *JCκ* deleted mice lack intronic enhancer elements as well as any *κ* locus or *RS* recombination (Chen et al., 1993). Third, in *Cκ* exon targeted mice, in which *Vκ*-to-*Jκ* recombination but not *RS* recombination occurred, λ B cell production was indistinguishable from that of mice unable to recombine any *κ* loci (Zou et al., 1993; Chen et al., 1993). These considerations argue against a simple model in which competition from recombinationally active *κ* loci suppresses *λ* recombination.

Our findings suggest that the defect in *λ* recombination seen in *RS* mutant mice occurred selectively in those B cells that expressed functional κ-chain. Analysis of BM B cells using intracellular staining for Igκ indicated that immature B cells expressing λ largely lacked any detectable κ expression and vice versa. Moreover, the extent of reduction of λ B cell production in *RS* mutant mice (~42%) was similar to the proportion of cells predicted to normally extinguish in-frame *κ* genes by *RS* recombination (Retter and Nemazee, 1998) and few *VκJκ5* junctions cloned from sorted λ^+ B cells of *RS−/ −* mice had in-frame κ rearrangements. In contrast to *κ* knockout mice, in *RS−/ −* mice λ generation is significantly, but partly suppressed, both in the presence or absence of κ-macroself Ag. Moreover, the reduced output of λ cells in *RS−/ −* mice is reflected in the amount of *λ* recombination excision circles. That almost all λ B cells that do develop in *RS* mutant mice lack κ expression appears to result from specific inhibition of *Igλ* recombination in sIgκ$^+$ cells, rather than counterselection of κ/ λ double positive cells after their formation.

An appealing alternative hypothesis to explain the dearth of λ B cell production in *RS* mutant mice is that induction of *λ* 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* mutant mice arises from their inability to silence κ protein expression in editing cells. This hypothesis is supported by the findings that relatively few λ/κ double expressing cells are generated in the BMs of *κ-macroself Ag* expressing mice in which all κ cells are autoreactive and induced to carry out receptor editing, even when survival was artificially enhanced by introduction of the *Bcl2* transgene. It appears that *λ* gene activation usually occurs selectively in cells lacking sIg, which occurs after *RS* mediated editing prevents κ expression. The relative inability of cells to undergo *λ* recombination when unable to silence expression of an autoreactive *κ* gene could occur if (a) immature autoreactive cells express lower recombinase levels than do sIg negative (preB) cells, (b) recombinase levels are limiting for editing and (c) *λ* gene recombination requires higher levels of recombinase than does *κ* recombination. There is evidence to support all of these possibilities. In early analyses of receptor editing in autoantibody transgenic mice, where virtually all developing B cells carried an autoreactive BCR, BM RAG mRNA levels, though high in the presence of autoantigen, were lower than in non transgenic controls dominated by small, sIg$^-$ preB cells (Tiegs et al., 1993; Lang et al., 1996). Moreover, RAG expression in BM B cells of *RS−/ −;Bcl2 Tg→ κ-macroself Tg* chimeras is reduced compared to *RS+/+* sufficient controls (Fig S4).

Furthermore, we have shown in two contexts that *RAG1* heterozygous deficiency suppresses markedly receptor editing in vivo (Verkoczy et al., 2005; Aït-Azzouzene et al., 2005), indicating that RAG expression is limiting for editing. Finally, λ genes are known to be much less efficiently recombined than κ genes because their recombination signals contain more non-consensus substitutions (Ramsden and Wu, 1991). Although the recombination signal of the *RS* element diverges from consensus to a similar extent as λ elements, *RS* mainly joins with *Vκ* elements, which carry near consensus recombination signals (Table 2). *RS* recombination usually correlates with λ recombination, and proceeds after initiation of κ rearrangements, however, exceptions 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), indicating that λ locus recombination can sometimes precede κ recombination. These exceptions might arise because λ genes assemble best in developing B cells that by chance or mutation fail to generate κ protein, regardless of their *RS* recombination status. We would therefore suggest that in autoreactive B cells undergoing receptor editing (and that are therefore sIg^+), RAG levels are limiting and are sufficient to drive κ and *RS* recombination, but generally insufficient to drive λ recombination, which we propose occurs preferentially after destructive editing first renders the cell sIg negative.

Experimental Procedures

Generation of *RS* knockout construct and production of knockout mice

Please see Supplementary data and experimental procedures.

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 (*Bcl2Tg*) mice (Strasser et al., 1991), were provided by Drs. Strasser and Harris (WEHI, Melbourne, Australia). *κ-macroself* transgenic (line 2) was described (Aït-Azzouzene et al., 2005). *RS*−/− mice analyzed had been backcrossed ten times to C57BL/6J mice (B6) and were compared to B6 controls. In some experiments involving mice carrying *κ macroself* and *Bcl2* transgenes had been backcrossed 6 times to B6 or B6.CD45.1. *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 Maxisorb 96 well plates in 0.5X Reacti-Bind DNA coating solution (Pierce). After overnight coating, wells were blocked for 1 hr in Tris buffered saline containing 5% non-fat dry milk powder. Mouse sera diluted in blocking solution supplemented with 1% 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 using a Versamax plate reader (Molecular Devices). Anti-dsDNA antibody concentration was normalized to a high titer control serum kindly provided by Dr. D. Kono. Flow cytometry data collection was done with an LSRII flow cytometer (Becton Dickenson) and analyzed using FlowJo software. For intracellular staining, surface stained cells were fixed and permeabilized using 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 post reconstitution recipients were sacrificed and their lymphoid

tissues analyzed. Only chimeras in which ≥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 followed by passage through LS columns (Miltenyi Biotec). Unbound cells were collected. Splenic B cells were similarly isolated but omitting the use of IgD depleting antibodies. The purity of these preparations was >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, 0.78 ng of B cell or spleen cell genomic DNA. The *Vλ1*-to-*Jλ1* excision product DNA rearrangements were done using the oligonucleotides and PCR conditions as described (Tiegs et al., 1993). *RS*-to-*IκRS* and *RS*-to-*V*&*kappa;* PCR assays were performed using primers B and C (Retter and Nemazee, 1998) along with a *Vκ* degenerate primer (Schlissel and Baltimore, 1989). Samples were amplified 25–30 cycles 1 min at 94° C, 1 min at 60°, and 1 min at 72°. 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 using a Phosphorimager using ImageQuant software (Molecular Dynamics).

Sequencing of *Vκ*l*Jκ5* light chains

Igλ$^+$ splenocytes were magnetically sorted using anti-Igλ1-3 biotinylated antibody and anti-biotin microbeads (Miltenyi) followed by cell sorting (FACSAria, BD). Bead isolated cells were stained with anti-Igκ (187.1 Alexa 647), CD4 PerCP-Cy5.5, CD3 PerCP-Cy5.5, CD19 PE-Cy7, B220 PacificBlue and streptavidin PE to detect the λ cells. Sorted λ$^+$/κ− B cells were confirmed to be >98% Igλ$^+$. DNeasy kit was used to isolate genomic DNA from ~2 million cells/ sample. *Vκ* degenerate and *Jκ5* primer (5′-TGCCACGTCAACTGATAATGAGCCCTCTCC-3′) were used for PCR amplification of *Vκ-Jκ5* rearrangements. The PCR products were electrophoresed on 1% agarose gels, purified using a kit (Qiagen), cloned in PCR4-TOPO plasmid vector (Invitrogen), and the inserts sequenced (Eton Bioscience). Sequence analysis was carried out with the Ig Blast program (http://www.ncbi.nlm.nih.gov/igblast/).

Hybridoma generation and analysis, antibody assays

B220$^+$ spleen cells were cultured for 48 hours in IMDM supplemented with 10% FCS and LPS (50 μg/ml) and fused with the SP2/0 myeloma line using polyethelene glycol. Cells from each fusion were then plated into 96-well plates and hybrids selected using HAT medium (ATCC). Due to 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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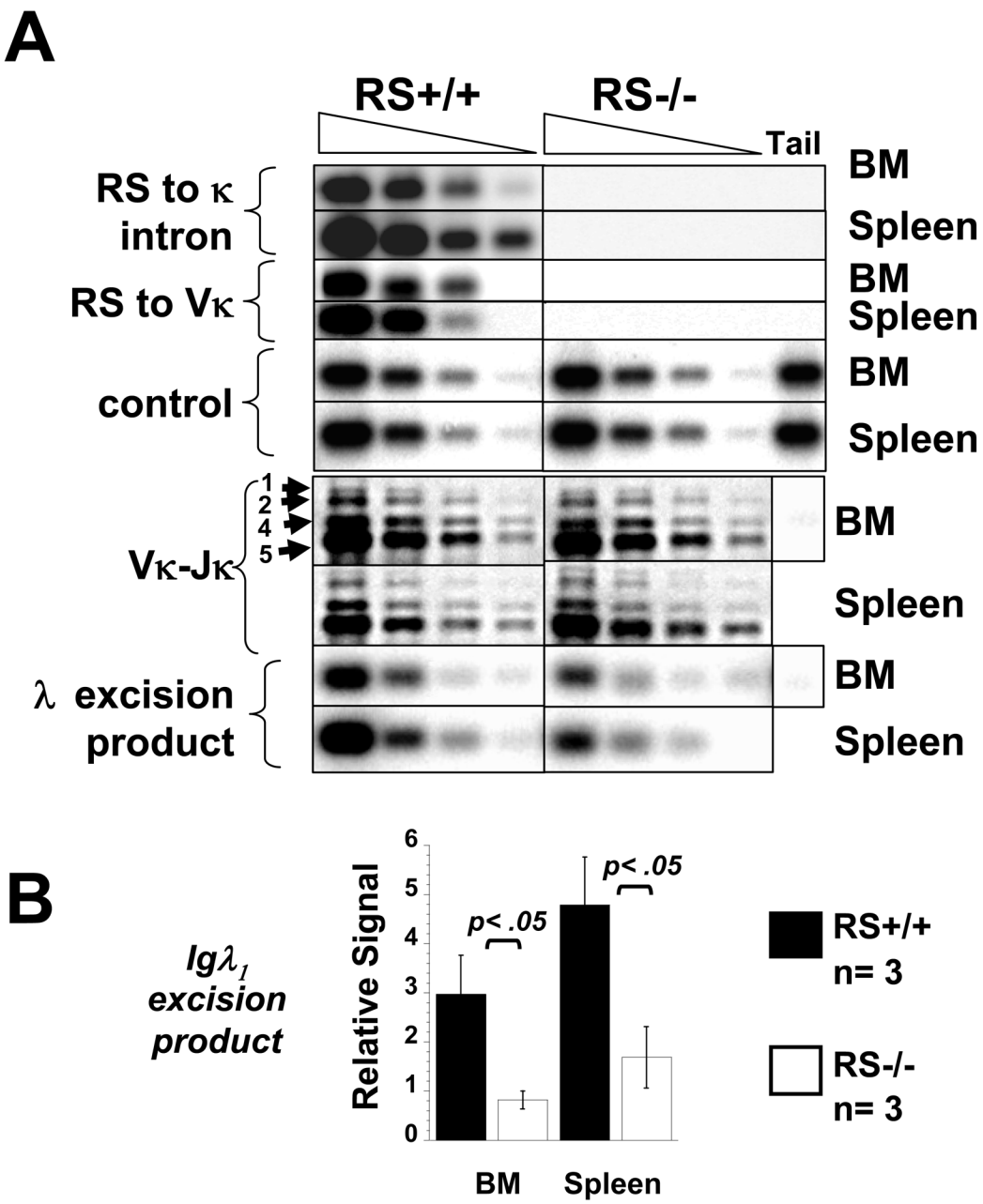

Figure 1.

Evaluation of *RS* recombination in spleen and BM B220$^+$ cells of *RS*−/− and littermate *RS*+/+ mice. Four-fold serial dilutions of the indicated DNA samples were subjected to PCR reactions to detect the indicated DNA rearrangements. PCR products were electrophoresed on agarose gels, blotted, and abundance quantitated by southern blot using 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 is 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 λ excision product levels in a sample of 3 mice/group.

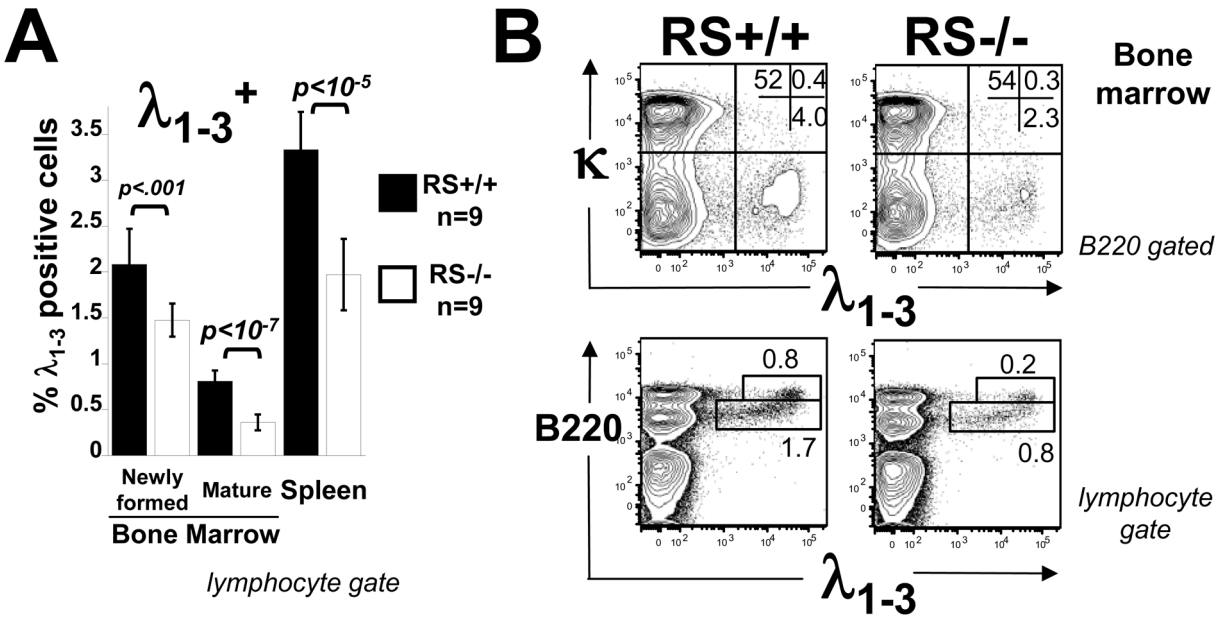

Figure 2.

Evaluation of reduced λ B cell frequencies in *RS* mutant mice. B cells from the BM and spleens of *RS*−/− and *RS*+/+ 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. **B)** Upper plot shows costaining for κ and λ in BMs of the indicated mice. Lower panels, gating used in A to distinguish newly formed from recirculating sIg^{+} B cells in BM.

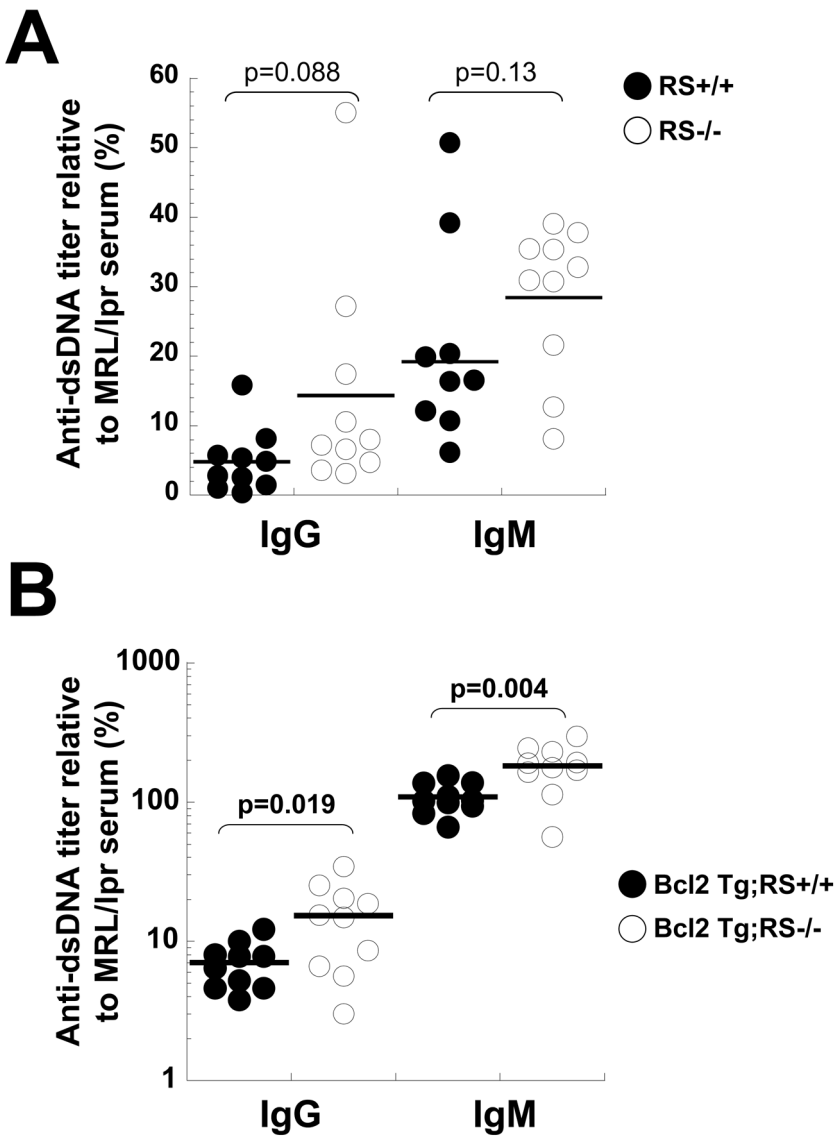

Figure 3.

Analysis of anti-dsDNA antibody in sera of the *RS* mutant and wild type mice. **A)** Anti-dsDNA titers in seven month old *RS*+/+ and *RS*−/− mice. **B)** Anti-dsDNA titers in 5 month old *Bcl2 Tg:RS*+/+ and *Bcl2 Tg;RS*−/− mice. Each dot represents the value obtained from a different individual mouse.

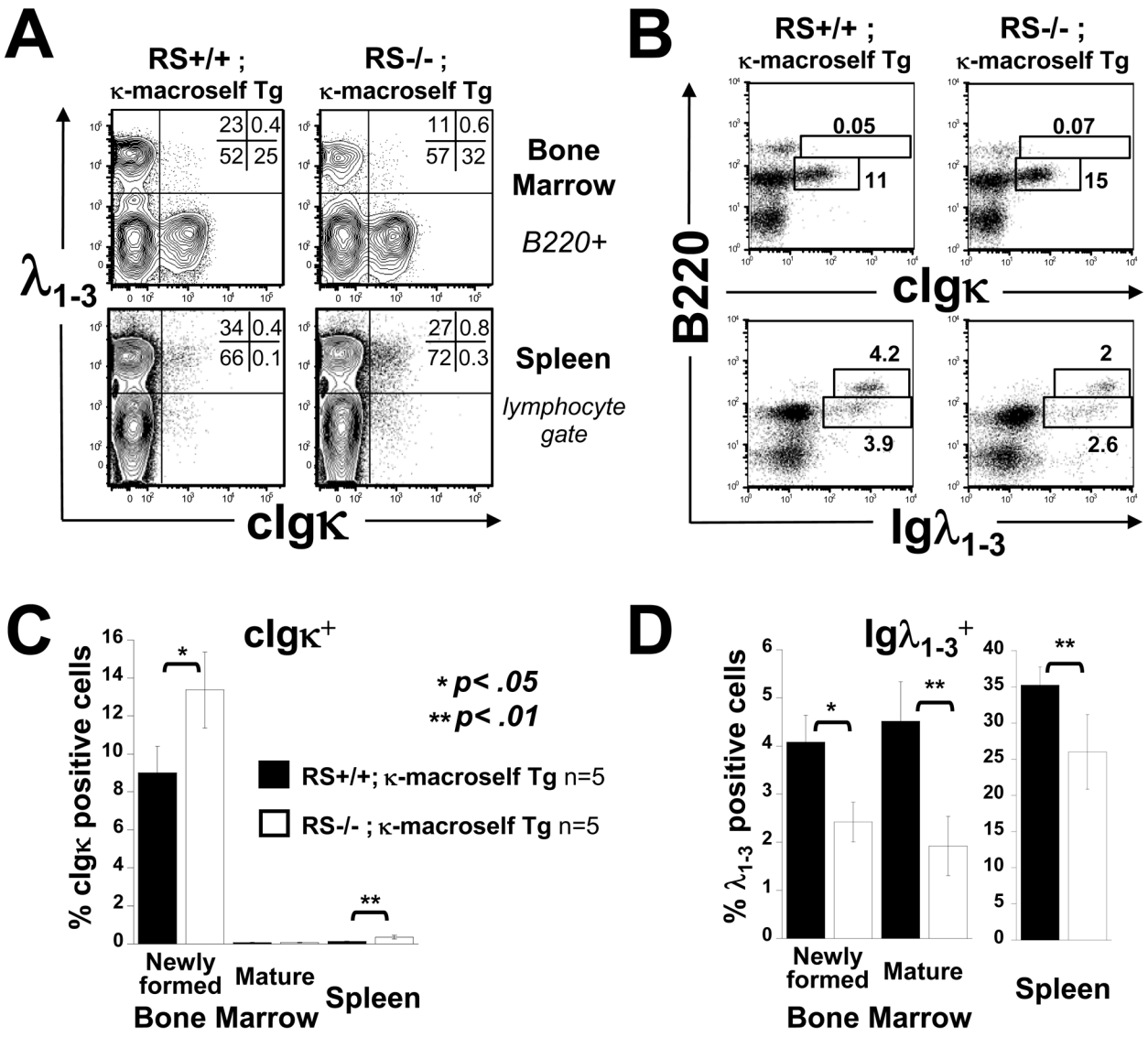

Figure 4.

Analysis of IgL-chain expression of *RS* mutant B cells developing in the presence of a κ superantigen. **A-D)** 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.

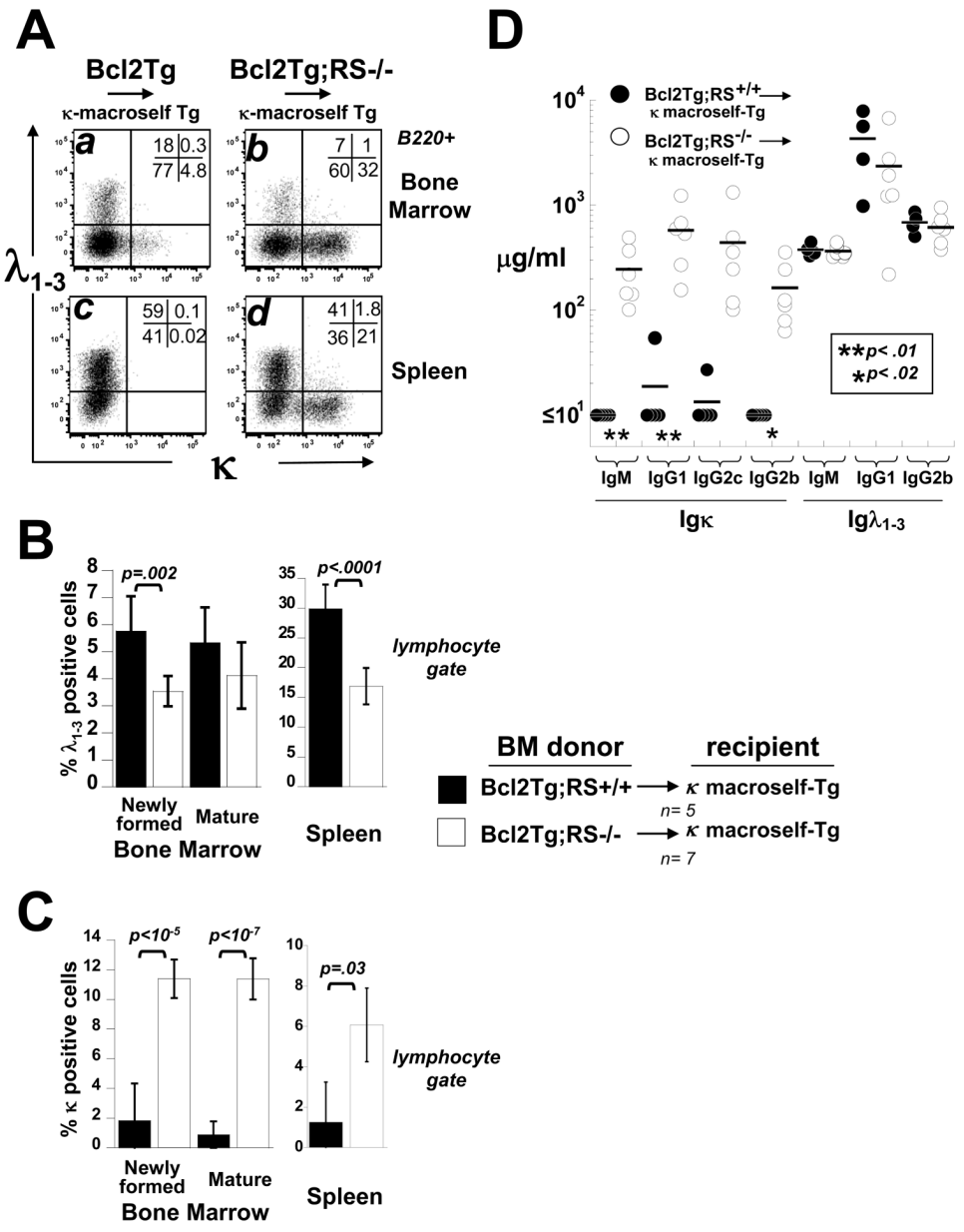

Figure 5.

Analysis of the effect of *RS* mutation on B cell tolerance in apoptosis-resistant cells. BM chimeras were generated using 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 post 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 Fig. 4A.) **B,C)** Summary analysis of the κ and λ frequencies of total splenic cells, and newly formed ($B220^{intermediate}$) and recirculating B cells ($B220^{high}$) from the indicated chimeras. Note increased κ frequency and reduced λ frequency among BM and spleen cells of κ-macroself recipients of *Bcl2 Tg;RS−/−* bone marrow. **D)** Igκ and Igλ serum immunoglobulin levels measured in the indicated chimeric mice.

Table 1

Analysis of B cell hybridomas from *RS*−/− and *RS*+/+ mice for λ_{1-3} secretion, κ/λ_{1-3} protein coproduction, and *RS* recombination.

Fusion number[a]	Mouse genotype	λ_{1-3}(%)	λ+/ #screened	λ+/κ+	λ+/κ	Total λ+ frequency	*RS* status of λ+ hybrids #rearranged / #tested	[b] *RS* recombination in λ^+ hybridomas
1	***RS*+/+**	7.4	2/27	0	2	**9 % (22/244)**	1/1	**100% (8/8)**
2		9	20/217	0	20		7/7	
3	***RS*−/−**	2.2	4/180	0	4	**2.8% (17/603)**	0/4	**0% (0/9)**
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 using spleen cells from a different individual mouse. Hybridoma clones were screened for κ and λ_{1-3}immunoglobulin production as indicated in 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.

Table 2

Analysis of recombination signal sequences of mouse antibody L chain loci relative to usage.

	9-mer	7-mer	non-consensus substitutions	approximate usage
Consensus	GGTTTTTGT	CACTGTG		
Jk1	---------	-------	0	33%
Jk2	a--------	-------	1	25%
Jk4	---------	-------	0	13%
Jk5	---------	-------	0	28%
Vk	---------	-------	0–2 (Avg 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 Coleclough, 1984; Durdik et al., 1984; Eisen and Reilly, 1985; Nadel et al., 1990; Shimizu et al., 1991; Luning Prak et al., 1994; Dunda and Corcos, 1997).