RS rearrangement frequency as a marker of receptor editing in lupus and type 1 diabetes

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Continued antibody gene rearrangement, termed receptor editing, is an important mechanism of central B cell tolerance that may be defective in some autoimmune individuals. We describe a quantitative assay for recombining sequence (RS) rearrangement that we use to estimate levels of antibody light chain receptor editing in various B cell populations. RS rearrangement is a recombination of a noncoding gene segment in the k antibody light chain locus. RS rearrangement levels are highest in the most highly edited B cells, and are inappropriately low in autoimmune mouse models of systemic lupus erythematosus (SLE) and type 1 diabetes (T1D), including those without overt disease. Low RS rearrangement levels are also observed in human subjects with SLE or T1D.

B cells undergo a random process of V(D)J recombination to generate the many distinct receptors needed to recognize a vast array of antigens. An inevitable consequence of this random process is the production of autoreactive B cells (1). An important mechanism for tolerizing autoreactive B cells is receptor editing (2). Receptor editing results in the alteration of B cell receptor specificity and is achieved by ongoing Ig gene rearrangement, most commonly at the light chain loci (3–5). Light chain rearrangement proceeds in an ordered fashion as B cells develop in the bone marrow, with k genes recombining first, followed by rearrangement of the recombining sequence (RS) and l (6, 7). The RS (also known as the k deleting element [KDE] in humans) is a noncoding gene segment located 25 kb downstream of Ck in the κ locus that is rearranged during continued Ig light chain gene rearrangement (8, 9).

Because of the unique structure of the κ locus, primary Vk-Jκ rearrangements that are nonfunctional or autoreactive can be replaced via “leap-frogging” recombination of unrearranged upstream Vk and downstream Jκ gene segments to form new κ light chains (Fig. 1 a). Additional rearrangement attempts can be made through recombination at the second κ allele or at l. Recombination of RS to upstream Vk gene segments or a recombination signal sequence within the Jκ-Cκ intron results in the deletion or inversion of Cκ and functional inactivation of the κ locus (Fig. 1 a). Because RS rearrangements do not encode any functional proteins (10), monitoring RS rearrangement provides a specificity-independent means of measuring repeated rearrangement attempts at κ (receptor editing).

The original studies characterizing RS recombination postulated that it served to promote l rearrangement by either repressing κ rearrangement or activating the l locus (7, 11). However, l-expressing B cells can form without undergoing RS rearrangement, indicating that RS is not required for the production of l (12). When RS rearrangement is prevented in RS knockout mice, receptor editing is inefficient and autoreactive B cells are found among peripheral cells (13), highlighting the potential role of RS in establishing central tolerance and reducing light chain allelic and isotypic inclusion.

Current clinical assays that evaluate B lymphocyte tolerance focus on serum autoantibodies, which are products of mature B cells. Because secreted autoantibodies are an end product rather than an intermediate, they do not distinguish between autoimmunity that arose...
during primary B cell maturation or later because of events such as somatic mutation. The distinction is important because a defect in primary B cell tolerance may predict disease development. Furthermore, diseases occurring as a result of a primary B cell tolerance defect may be associated with resistance to B cell–targeted therapy because the primary repertoire is predicted to rapidly repopulate with autoreactive cells if B cell reconstitution is allowed to proceed. Before testing those ideas, an assay for central B cell tolerance is needed.

This manuscript describes the development and initial characterization of RS rearrangement frequency as an assay for central B cell tolerance in systemic lupus erythematosus (SLE) and type 1 diabetes (T1D). In both of these diseases, B cells play a critical pathogenic role. Autoantibodies are a prominent feature, whether they be directed against nuclear antigens (in SLE) (14), or pancreatic β-cell antigens such as GAD65 or insulin (in T1D) (15). Furthermore, B cell deficiency or depletion in mouse models ameliorates or prevents disease (16–20). The results with B cell depletion therapy in humans are equivocal for SLE (EXPLORER study, available at http://www.clinicaltrials.gov under identifier no. NCT00137969) and not yet fully explored in T1D. In this paper, we describe the status of central tolerance in individuals with SLE or T1D using RS rearrangement frequency as a marker.

RESULTS

A novel assay for estimating levels of receptor editing
We focused on RS rearrangement as an assay for receptor editing because RS rearrangement is known to accompany extensive light chain rearrangement during primary B cell maturation (11, 21), and the major RS rearrangement products are defined and share a common DNA sequence (22).

Figure 1. RS rearrangement is a marker of extensive κ light chain rearrangement. (a) Schematic of the mouse κ light chain locus illustrating successive Vκ-Jκ rearrangements followed by RS rearrangement. Two pathways of RS rearrangement are available. The first (1) involves recombination of an upstream unrearranged Vκ gene segment to RS, whereas the second (2) utilizes a noncanonical recombination signal sequence (iRS) in the Jκ-Cκ intron to rearrange to RS. Both result in the deletion of Cκ and functional inactivation of the Igκ locus. Exons are indicated by boxes, recombination signals are indicated by triangles, and dashed lines with arrows illustrate the rearrangements. (b) RS rearrangement levels as measured in Igκ+ (gray bars) and Igκ− (presumed to be κ−; black bars) from splenic B220+ IgM+ B cells of adult (3–4-mo-old) C57BL/6 mice (n = 5) and CD19+ 9G4− peripheral B cells from healthy control subjects (n = 26). All PCR reactions were performed in duplicate. Mouse data are presented as the fold difference relative to the mean RS level in C57BL/6 splenic B220+ IgM+ Igκ+ B cells (+SEM). Human data are depicted as rearrangement frequency per genome copy (+SEM).
Most importantly, RS rearrangements do not encode a functional protein (10) and, therefore, are independent of antibody specificity, making their measurement potentially applicable to any disease in which B cells play a pathogenic role. By combining a quantitative PCR assay for RS rearrangement frequency with cell sorting, receptor editing can be analyzed in different B cell subsets (Fig. S1, available at http://www.jjem.org/cgi/content/full/jem.20082053/DC1).

We analyzed the most abundant class of RS rearrangements, which are \( V_k \) to \( RS \) in mice and intron \( RS \) (iRS) to \( RS \) in humans (23, 24). For the human studies, iRS to \( RS \) rearrangements were quantified relative to an absolute standard consisting of a cloned iRS-RS rearrangement that was serially diluted in fibroblast DNA. Log-linear amplification was observed over the range of 0.7–200% RS rearrangements per cell genome (Fig. S2, available at http://www.jjem.org/cgi/content/full/jem.20082053/DC1). 200% corresponds to having two RS rearrangements, one on each \( \kappa \) allele. Typical RS frequency measurements fall within this log-linear range.

For the mouse studies, a degenerate \( V_k \) primer was used for RS rearrangement measurements (25). Mouse \( V_k \)-RS rearrangements were quantified as fold difference relative to IgM+ \( \kappa^* \) spleen DNA from B6 mice (the spleen contains a mixture of different \( V_k \)s). Fold differences were used rather than absolute frequencies to avoid confusion caused by differing amplification efficiencies for different \( V_k \) genes.

The correlation between RS rearrangement and extensive light chain rearrangement has been established in earlier works from several groups (8, 9, 23, 24, 26, 27). Consistent with these earlier studies, RS rearrangement is increased eightfold among \( \lambda^+ \) mouse IgM+ \( \kappa^* \) B cells compared with \( \kappa^* \) cells (Fig. 1 b). Among CD19+ human B cells, 16% of \( \kappa^* \) B cells carried an RS rearrangement, and the frequency of RS rearrangements among \( \lambda^+ \) B cells was >100%, indicating that some \( \lambda^+ \) B cells have rearranged to RS on both \( \kappa \) alleles. Because RS levels are higher in \( \lambda^+ \) than in \( \kappa^* \) B cells, the overall RS rearrangement frequency correlates inversely with the \( \kappa/\lambda \) ratio. To reduce the variability in RS frequency measurements introduced by the \( \kappa/\lambda \) ratio, RS analysis was performed separately in \( \kappa^* \) or \( \lambda^+ \) cells. However, it is important to note that RS rearrangement levels in a given \( \kappa^* \) or \( \lambda^+ \) B cell population vary independently from the overall \( \kappa/\lambda \) ratio (Fig. S3, available at http://www.jjem.org/cgi/content/full/jem.20082053/DC1; and see Fig. S5 a). Thus, measuring the RS frequency is not simply a cumbersome method for assessing the \( \kappa/\lambda \) ratio. Also of note, RS rearrangement frequencies are correlated in \( \kappa^* \)- and \( \lambda^+ \)-expressing B cells within single individuals (this intrapersonal correlation and the concept of an RS set point will be addressed in the analysis of human subjects that follows).

RS rearrangement levels vary between developmental and functional B cell subsets in mice

If RS rearrangement is a marker of ongoing antibody light chain rearrangement, it should occur at the time of late \( \kappa \) or \( \lambda \) rearrangement. Consistent with this prediction, the highest level of RS rearrangement was found among bone marrow fraction (Fr.) D cells (small pre-B II cells [28]), which are cytoplasmic IgM+ but no longer express surrogate light chain. Fr. D cells express the Rag1 and Rag2 proteins for light chain gene rearrangement (Fig. 2 a) (28). Further subsetting within Fr. D based upon BP-1 (aminopeptidase A) (BP-1) antibody staining revealed that the majority of RS rearrangements most likely occurred toward the end of this period (Fig. 2 b).

In the subsequent developmental subset, Fr. E (newly formed IgM+ immature B cells), \( V_k \)-RS rearrangement levels were reduced by threefold and were closer to those of mature, circulating B cells (Fr. F). The difference in \( V_k \)-RS rearrangement levels between Fr. D cells and \( \kappa^* \) Fr. E cells could be
caused by the inclusion of pre–B cells undergoing or completing A rearrangement within Fr. D. However, RS levels in total IgM+ Fr. E cells were similar to κ+ Fr. E cells, suggesting that exclusion of κ+ cells from Fr. E cannot fully account for the decrease. Death of RS+ cells or their rapid exit from the bone marrow to a peripheral pool may contribute to the decrease in RS rearrangement levels in Fr. E relative to Fr. D.

RS rearrangement levels differ between autoreactive and nonautoreactive B cells

It is unclear from the preceding experiments how or if RS rearrangement frequency is linked to autoreactivity. Editing is driven either by the potential of the antibody heavy light chain pair to form autoantibodies (active model), or it occurs without regard for receptor specificity (passive model). To distinguish between active and passive editing, RS rearrangement frequencies were analyzed in the 56R mouse model, where the B cell repertoire has been characterized extensively (29–31). The 56R mouse carries a site-directed transgene encoding an anti-DNA–specific Ig heavy chain. Using B6.56R+/− mice, one can distinguish B cells with an autoreactive Ig heavy chain (IgMκ+, mostly 56R expressing) and endogenous (IgMκ−, 56R−) B cells (Fig. 3 a). 56R–expressing B cells have a restricted light chain repertoire consisting of only a handful of light chains, termed editors, because they modify or reduce DNA binding (29–31). This restriction in light chain usage could arise if 56R B cells with noneditor light chains were counterselected or if B cells with noneditor light chains were subjected to more receptor editing. The former alternative predicts equal levels of RS rearrangement in IgMκ+ and IgMκ− cells, whereas the latter predicts higher levels of RS rearrangement in IgMκ+ B cells. Consistent with an active model of receptor editing, a twofold increase in RS rearrangement levels was observed in IgMκ+ B cells (Fig. 3 b).

Mouse strains prone to autoimmunity display lower levels of RS rearrangement

The preceding experiments established the developmental timing of RS rearrangement and documented a positive correlation between RS rearrangement frequency and an autoreactive antibody heavy chain in inbred mice. To determine if RS rearrangement is altered in the context of autoimmunity, we measured RS rearrangement frequency in two different mouse models of autoimmune disease, MRL/lpr mice as a model of SLE and nonobese diabetic (NOD) mice as a model of T1D. We observed decreases in RS rearrangement levels among Fr. D cells of both MRL/lpr (2.5-fold reduction; P < 0.05) and NOD (3.7-fold reduction; P < 0.01) mice when compared with C57BL/6 mice (Fig. 4 a). Interestingly, fewer RS rearrangements were also observed among Fr. E cells in both MRL/lpr and NOD mice relative to C57BL/6 mice. This decrease was evident in the mature circulating B cells of the bone marrow (Fr. F) as well, implying that unedited or minimally edited cells persisted through development. Measurement of RS levels in splenic B cell subsets further substantiated this finding (Fig. 4 b).

To determine if the lower levels of Vκ-RS rearrangements detected in MRL/lpr and NOD mice are attributable to strain effects, rather than as consequences of an autoimmune state, RS levels were measured in MRL/MpJ mice, which share the same genetic background as MRL/lpr mice but lack the Fas mutation that is responsible for their lymphocytosis and accelerated systemic autoimmunity. Similarly, NOD mice were compared with nonobese resistant (NOR)
mice, which share the same diabetogenic MHC haplotype (H2^g7) with NOD mice but are insulin resistant and do not develop T1D (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20082053/DC1) (32). These “control” strains share the propensity to develop autoimmunity with MRL/lpr and NOD mice but do so at a much slower pace. The control mice were analyzed at 3 mo of age, a time at which autoimmune pathology is not yet apparent. Although RS rearrangement levels were slightly increased among some bone marrow B cell subsets in the control mouse strains, levels in mature B cells were comparable in MRL/MpJ and NOR mice to their more autoimmune counterparts. The low frequency of RS rearrangement in MRL/MpJ and NOR mice suggests that a reduced level of receptor editing predisposes toward the development of autoimmunity rather than arising as a consequence of autoimmune disease.

RS rearrangement levels are lower in human SLE and T1D To further investigate the level of receptor editing in the context of defective tolerance, we compared RS rearrangement levels in peripheral B cells from human subjects with established disease (SLE or T1D) to healthy control subjects (see Materials and methods). For this analysis, we evaluated 26 control subjects, 24 patients with SLE, and 25 patients with T1D. Demographic features of the subject groups were compared, and no significant differences were observed between control and SLE groups with respect to age, gender, and race; however, the T1D group contained a larger proportion of men and Caucasians than the control and SLE groups (Table I). Nonetheless, RS frequencies were not correlated with subject age, gender, or race (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20082053/DC1).

Among CD19^+ κ^+ B cells, RS rearrangement frequencies from SLE patients were lower on average compared with levels from healthy controls (P < 0.01; Fig. 5 a). The RS rearrangement frequency in κ^+ B cells from T1D was not significantly decreased compared with control subjects. In CD19^+ λ^+ B cells, RS levels were lower in both SLE and T1D patients compared with control subjects (P < 0.01; Fig. 5 b). These results resemble those from the autoimmune mouse models and suggest that lower levels of RS rearrangement may be correlated with disease susceptibility.

We chose the bottom 10th percentile of the normal population as an arbitrary cutoff for the RS rearrangement frequency distribution in κ^+ B cells (κRS). Using this cutoff, the fraction of subjects with a low κRS value is 2 out of 26 for the healthy control subjects, 13 out of 24 for SLE, and 8 out of 25 for T1D. Using a similar ARS cutoff, 3 out of 26 controls, 9 out of 23 SLE patients, and 11 out of 25 T1D patients had low RS levels. Additionally, RS levels were low in both κ^+ and λ^+ B cells in 7 out of 23 SLE and 7 out of 25 T1D patients, indicating that subjects with low κRS levels tended to also have low ARS levels. Indeed, Spearman correlations between the κRS and ARS measurements were significant in both healthy subjects and patients with autoimmune disease (r = 0.54, P < 0.01 and r = 0.52, P < 0.01, respectively; Fig. 5 c). Additionally, linear regression analysis revealed a lower degree of RS rearrangement in λ^+ cells relative to levels in κ^+ cells in patients with autoimmune disease compared with healthy controls. Collectively, these findings suggest that some SLE and T1D patients have lower editing set points than those of most healthy individuals.

DISCUSSION
The development of assays to monitor B cell tolerance and selection stringency can inform the choice of B cell–targeted therapy for autoimmune disease. This contention is based on the idea that individual patients with SLE and T1D have different tolerance defects (33, 34). Previous assays that monitor B cell tolerance are problematic because they tend to measure relatively late events, such as autoantibody production, rather than the pathways that B cells take on their way to becoming autoimmune. The RS rearrangement assay presented in this paper takes 2 d to perform and provides insights into central (early) B cell development of assays to monitor B cell tolerance and selection stringency can inform the choice of B cell–targeted therapy for autoimmune disease. This contention is based on the idea that individual patients with SLE and T1D have different tolerance defects (33, 34). Previous assays that monitor B cell tolerance are problematic because they tend to measure relatively late events, such as autoantibody production, rather than the pathways that B cells take on their way to becoming autoimmune. The RS rearrangement assay presented in this paper takes 2 d to perform and provides insights into central (early) B cell development. We chose the bottom 10th percentile of the normal population as an arbitrary cutoff for the RS rearrangement frequency distribution in κ^+ B cells (κRS). Using this cutoff, the fraction of subjects with a low κRS value is 2 out of 26 for the healthy control subjects, 13 out of 24 for SLE, and 8 out of 25 for T1D. Using a similar ARS cutoff, 3 out of 26 controls, 9 out of 23 SLE patients, and 11 out of 25 T1D patients had low RS levels. Additionally, RS levels were low in both κ^+ and λ^+ B cells in 7 out of 23 SLE and 7 out of 25 T1D patients, indicating that subjects with low κRS levels tended to also have low ARS levels. Indeed, Spearman correlations between the κRS and ARS measurements were significant in both healthy subjects and patients with autoimmune disease (r = 0.54, P < 0.01 and r = 0.52, P < 0.01, respectively; Fig. 5 c). Additionally, linear regression analysis revealed a lower degree of RS rearrangement in λ^+ cells relative to levels in κ^+ cells in patients with autoimmune disease compared with healthy controls. Collectively, these findings suggest that some SLE and T1D patients have lower editing set points than those of most healthy individuals.

**Figure 4.** Autoimmune-prone mouse strains display reduced levels of RS rearrangement. (a) Vκ-RS rearrangement levels in bone marrow subsets of MRL/lpr mice (white bars; n = 4) and NOD mice (gray bars; n = 5). Data from C57BL/6 mice are included for comparison. (b) Vλ-RS rearrangement levels in κ^+ splenic B cell subsets from C57BL/6 (black bars; n = 5), MRL/lpr (white bars; n = 4), and NOD (gray bars; n = 5) mice. All PCR reactions were performed in duplicate. Data are represented as fold difference (+SEM; *, P < 0.05 and **, P < 0.01) relative to the mean RS level in C57BL/6 splenic B220^+ IgM^+ κ^+ B cells (dashed lines).
cell tolerance. Furthermore, it can be combined with immunophenotyping to analyze editing levels in different peripheral B cell subsets. As such, it may be possible in the future to use this assay to monitor and stage defects in B cell tolerance.

The RS rearrangement assay gives an estimate of the overall level of light chain rearrangement in a defined population of B cells. The correlation between RS level and the light chain rearrangement level is based on earlier studies documenting that 10–15% of mouse k+ splenic B cells had undergone RS recombination, whereas virtually all λ+ splenic B cells harbored an RS rearrangement (23, 26, 27). Similarly, studies of single human B cells and acute lymphoblastoid leukemia cell lines demonstrated that cells expressing λ harbored more RS rearrangements than ones that expressed k (24, 35). Furthermore, λ B cells took ~24 h longer to label with BrdU than B cells with k rearrangements (36). Finally, studies in B cell leukemia cell lines subjected to Bcr-Abl tyrosine kinase blockade exhibited temporally ordered light chain rearrangements: k followed by RS followed by λ (21). The current study is consistent with all of these previous studies: RS rearrangement frequencies were higher in λ+ B cells than in k+ B cells and were most frequent in late pre-B cells (Fr. D). Every other B cell subset, including Fr. E (where surface antibody is first detected) and more mature splenic B cells, exhibited lower RS levels. As RS rearrangements are nonrevertible, these findings suggest that receptor editing fails to salvage some of the most highly edited B cells from clonal deletion. An increased RS rearrangement frequency was also observed in B cells with autoreactive heavy chains. Because RS rearrangements do not encode a protein that can be selected, this result indicates that the presence of an autoreactive heavy chain increases the likelihood of ongoing light chain rearrangement and is consistent with other reports favoring an active, autoantigen-driven model of receptor editing (37, 38). These findings are consistent with earlier work in autoantibody transgenic and knock-in mice showing that some highly edited B cells are auto-/multireactive (39–42). The loss of B cells with more RS rearrangements could also reflect the failure to produce a functional antibody.

The current study demonstrates decreases in RS rearrangement frequency in mouse models of SLE and T1D. Both MRL/lpr and NOD mice exhibited lower RS levels compared with wild-type C57BL/6 mice. The finding of lower editing levels in MRL/lpr mice is consistent with recent work from Lamoureux et al. demonstrating decreased receptor editing in response to a ubiquitous membrane-bound self-antigen in transgenic MRL/lpr mice (43). The finding of decreased RS rearrangement in NOD mice is at odds with a previous report in which editing was measured using a membrane-bound facultative self-antigen (hen egg lysozyme) (44). The RS assay, however, does not focus on one kind of self-antigen.

Although both MRL/lpr (45) and NOD (46) strains harbor previously characterized defects in apoptosis, lower RS levels were not linked to these defects per se, as B cells from MRL/MpJ and NOR mice (both with intact apoptosis) also exhibited lower RS levels. In the case of MRL/lpr mice, the additional defect conferred by the lpr mutation (45) may lead to less stringent selection of minimally edited immature B cells. Similarly, the finding of low RS rearrangement levels in both the NOD and NOR strains suggests that a second tolerance defect present only in NOD mice is required for development of diabetes. However, the genetic backgrounds of these control strains predispose them to develop autoimmunity. Despite having an intact Fas gene, MRL/MpJ mice spontaneously develop autoimmunity, including pancreatitis (47) and glomerulonephritis (48), but the disease is milder and occurs later in life. NOR/LtJ mice share the diabetogenic H2k haplotype with NOD mice and have altered macrophage and peripheral T cell compartments (32). All of the mice used for these experiments were 3 mo of age, which is at a time that precedes disease development in NOR and MRL/MpJ mice. Collectively, these findings suggest that decreased RS rearrangement levels in these mice reflect an altered propensity to develop autoimmunity.

In humans, lower RS rearrangement levels were also detected in both SLE and T1D patients compared with healthy control subjects. The decrease was most apparent in λ+ B cells, but the level of RS rearrangement was correlated in k+ and λ+ B cells.

### Table I. Demographic comparison among subject groups

|                         | Control | SLE      | T1D      |
|-------------------------|---------|----------|----------|
| Number of subjects      | 26      | 24       | 25       |
| Average age (yr)        | 33.8 (22–52) | 37.3 (21–55) | 38.5 (19–69) |
| Female (%)              | 0.88    | 0.88     | 0.56     |
| Caucasian (%)           | 0.58    | 0.29     | 0.92     |
| African-American (%)    | 0.27    | 0.42     | 0.04     |
| Other (%)               | 0.15    | 0.29     | 0.04     |
| Mean k/λ ratio (CD19+)  | 1.5 (1.1–2.3) | 1.8 (1–4) | 1.6 (0.8–2.4) |
| Mean B cell fraction (CD19+) | 0.11 (0.05–0.19) | 0.1 (0.01–0.2) | 0.1 (0.02–0.19) |
| Mean absolute B cell count (per μl) | 221 ± 126 | 176 ± 163 | 190 ± 107 |

<sup>a</sup>Significant differences were found with respect to gender and race between the T1D and control groups (P < 0.05 and P < 0.01, respectively) as well as between the T1D and SLE groups (P < 0.05 and P < 0.001, respectively).

<sup>b</sup>Absolute B cell counts were obtained by multiplying the WBC count (from the complete blood count) by the percentage of lymphocytes (from the electronic differential) and by the CD19+ fraction, and are listed as means ± SD. Absolute B cell counts represent the average of 25 control subjects, 23 SLE patients, and 24 T1D patients, respectively.
B cells in individual patients. The levels were not proportional to the κ/λ ratio or to the fraction of B cells in the blood. These findings have theoretical and practical implications. The theoretical implication is that individuals may have different RS rearrangement "set points." The set points tend to be lower in T1D and SLE than in healthy subjects but overlap to some degree. It will be important to directly measure RS levels in different peripheral B cell subsets and determine if differences can be correlated with B cell selection checkpoints or if, in a fashion similar to the mouse models, different subsets will show correlated RS levels. Until now, selection checkpoints have been monitored by single-cell antibody cloning and expression studies (1, 49, 50). If RS rearrangement can be used instead, it may be possible to more easily define B cell tolerance checkpoints in individual patients with autoimmune disease. The practical implication of having correlated RS rearrangement frequencies in κ+ and λ+ B cells is that it may be possible to measure RS rearrangement frequency in whole blood rather than in sorted B cell subsets and then to correct the measurement for the B cell fraction and κ/λ ratio.

Given the concordance of RS rearrangement levels in T1D and SLE, we considered the possibility that upstream genetic defects could decrease receptor editing in both disorders. One candidate is protein tyrosine phosphatase nonreceptor 22 (PTPN22; also known as Lyp). PTPN22 is a lymphoid-specific phosphatase that suppresses T cell activation (51). A variant of PTPN22 (R620W) is common in both SLE and T1D (52, 53) and appears to have a more active phosphatase in the setting of T1D (54). Although the effects of PTPN22 R620W are not yet well defined in B cells (55), one is tempted to speculate that there could be an increased BCR signaling threshold caused by PTPN22 R620W that would result in decreased receptor editing. Other potential candidates include RAG or molecules that influence the intracellular localization or expression of RAG, such as the nuclear importin KPNA1 (56), IFN regulatory factor 4 (57), or the transcription factor Foxo1 (58, 59). Because RS rearrangement frequency corresponds to a phenotype rather than a heritable genetic alteration, there could be several different defects, each of which could contribute independently to a low RS frequency.

It is not yet clear what the biological consequences are of a low RS rearrangement frequency or if the lowest levels of RS rearrangement are inherently the most dangerous. Perhaps individuals with low RS levels (including a small subset of currently healthy subjects) are at increased risk of developing autoimmunity. In this regard, the prospective analysis of first-degree relatives of patients with autoimmunity might be especially informative. It will also be important to find out how generalizable the RS rearrangement frequency is to

Figure 5. RS rearrangement levels are lower among SLE and T1D patients. (a and b) iRS rearrangement frequencies as quantified in peripheral CD19+ 9G4+ κ+ and CD19+ 9G4− λ+ B cells, respectively. Data represent the iRS levels in healthy control subjects (black circles; n = 26), SLE patients (white squares; n = 24 for κ+ and n = 23 for λ+), and T1D patients (gray triangles; n = 25). Mean values are depicted as horizontal lines. The 10th percentile of iRS frequencies among control subjects is depicted as a dashed line. (c) iRS rearrangement frequencies in CD19+ 9G4+ κ+ peripheral B cells compared with frequencies in CD19+ 9G4− λ+ cells from healthy controls (black circles; n = 26) and SLE and T1D patients (white squares; n = 48). All PCR reactions were performed in duplicate. Data are depicted as iRS rearrangement frequency per genome copy. Trend lines are depicted for control patients (continuous line; slope = 5.4) and autoimmune patients (dashed line; slope = 3.3; r-values indicate Spearman correlation coefficients; **, P < 0.01).
other autoimmune diseases as a potential marker for defective central tolerance.

**MATERIALS AND METHODS**

**Mice.** B6.36R mice have been described previously (30). C57BL/6, NOD, NOR, MRL/lpr, and MRL/MpJ mice were obtained from the Jackson Laboratory. All animal experiments were performed on 3–4-mo-old mice in accordance with protocols approved by the University of Pennsylvania School of Medicine Animal Care and Use Committee.

**Flow cytometry.** Mouse cell suspensions were prepared from femurs, tibias, and spleens in FACS buffer (PBS, 0.5% BSA, 0.01% Na3NO, 1 mM EDTA) after hypotonic RBC lysis (AACK Lysing Buffer; BioWhittaker). B cell populations were defined as described in Fig. S1. Because of poor reactivity of AA4.1 antibodies in NOD and NOR mice (60), bone marrow and peripheral subsets in these mice were defined using a method that has been described previously (Fig. S1) (61). Human B cells were isolated from whole blood using Lymphocyte Separation Medium (MP Biomedicals) followed by resuspension in FACS buffer. Mouse lymphocytes were stained using anti-IgM-PE-Cy7 (II/41), anti-IgD-PE (11-26), anti-B220-allophycocyanin (APC)-AF750 (RA3-6B2; eBioscience), and anti-Igk-FITC (187.1), anti-BP-1-FITC (6C3), anti-CD43-PE (S7), anti-CD23-PE (BB4), and anti-AA4.1-APC (BD). Human lymphocytes were stained with anti-CD19-Pacific blue (Invitrogen) and anti-Igκ-FITC, anti-CD27-FITC, anti-Igκ-PE, and anti-CD38-PE-Cy7 (BD). To maximize efficient use of patient samples for additional experiments not described in this paper, CD19+ B cells marked by the anti-idiotypic monoclonal antibody 9G4 (provided by M. Sanders, Paliggen, Inc., Palo Alto, CA), which recognizes V_{H}4-34 heavy chain rearrangements (62), were excluded from the human CD19+ B cell populations described (Fig. S1 d). 9G4Id+ cells comprised 5.1%, 5.7%, and 4.2% of total CD19+ B cells from control, SLE, and T1D subjects, respectively. All cells were sorted using a FACSAria (BD), with sort purities >90%.

**Quantitative PCR.** Genomic DNA was isolated from sorted B cells using the Gentra Puregene Tissue Kit (QIAGEN). Quantitative PCR (40°C for 10 min, 95°C for 10 min, followed by 60 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s) was performed on 15–50-ng template DNA in a 20-μl reaction mix containing 1× LightCycler 480 Probes Master Mix (Roche), 0.5 U LightCycler Uracl-β-DNA Glycosylase (Roche), 0.5 μM of a forward primer, 0.5 μM of a reverse primer, and 0.2 μM of a hydrolysate probe using a LightCycler 480 real-time PCR system (Roche). Primers and probes are described in Fig. S2. The amount of Vκ-RS product in each mouse sample was normalized to the amount of β-actin product and compared with the normalized target gene in wild-type C57BL/6 B220+ Igκ+ splenocytes to determine a relative quantity (comparative Ct [ΔΔCt] method). The frequency of iRS-KDE rearrangements in each human sample was determined via absolute quantification using a standard curve generated by serial dilution of a cloned iRS-KDE rearrangement resuspended with 100 ng of human fibroblast DNA (Fig. S2). To determine the number of genome copies, β-actin was measured via absolute quantification using a standard curve consisting of serially diluted human fibroblast DNA. Reactions were performed in duplicate, and samples with inconsistent replicates or β-actin cycle numbers >35 were excluded.

**Human subjects.** Volunteer healthy adult subjects with no history of autoimmune disease, no active viral or bacterial infection, or current use of immunomodulatory or immunosuppressive drugs were recruited. Women who were nursing, pregnant, or planning on becoming pregnant during the time of this study were excluded. Adults with documented SLE (fulfilling the American Rheumatism Association criteria [63]) were recruited from the Rheumatology Clinic at the Hospital of the University of Pennsylvania. Blood draws from SLE patients receiving steroids were performed before their first morning dose. T1D patients were recruited from the Rodebaugh Diabetes Center at the Hospital of the University of Pennsylvania. Subjects were included if they had a clinical history compatible with autoimmune T1D, defined by insulin dependence, the absence of obesity, and an age of onset <40 yr or >40 yr together with elevated levels of autoantibodies to GAD65. Subjects meeting these criteria but presently receiving immunosuppression drugs to support a kidney, pancreas, or islet cell transplant were excluded. The study protocol was approved by the Institutional Review Board of the University of Pennsylvania, and all subjects gave their written informed consent to participate.

**Statistical analysis.** Group comparisons for the mouse experiments were performed with a two-tailed Student’s t test unless otherwise specified. Group comparisons in human subjects were performed via one-way analysis of variance followed by a two-tailed Student’s t test. Correlation analyses were performed by calculating Spearman’s rank correlation coefficients. Categorical data (gender and race) comparisons were analyzed using Fisher’s exact test. For all tests, P ≤ 0.05 was considered significant.

**Online supplemental material.** Fig. S1 depicts flow cytometric analysis of mouse and human B cell populations isolated for RS quantitation. Fig. S2 describes the absolute quantification method used to monitor iRS-KDE rearrangements via real-time PCR. Fig. S3 demonstrates that RS rearrangement levels among κ+ B cells are independent of κ/λ ratios. Fig. S4 depicts RS rearrangement levels in MRL/MpJ and NOR mouse strains. Fig. S5 demonstrates that differences in iRS rearrangement levels among subject groups do not correlate with demographic differences. Online supplemental material is available at http://www.jemb.org/cgi/content/full/jem.20082053/DC1.

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