**Research article**

**B cell receptor revision diminishes the autoreactive B cell response after antigen activation in mice**

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**Introduction**

During B lymphocyte development in the BM, immature B cells expressing self-reactive antigen receptors are tolerated by a variety of mechanisms. Prominent among these are clonal deletion, which purges B cells reactive to self antigens from the repertoire (1), and induction of clonal anergy, which renders autoreactive B cells nonresponsive to B cell receptor (BCR) stimulation (1, 2). In addition, secondary variable (diversity) joining [V(D)J] rearrangement mediated by RAG1 and RAG2, which is termed receptor editing and occurs particularly at Ig light chain loci, helps maintain tolerance by modifying the specificity of the BCR (3, 4). The fate of individual autoreactive B cells is determined by multiple factors, including the strength of signaling through the BCR, the developmental stage at which BCR engagement by self antigen occurs, whether the self antigen is soluble or membrane bound, and the presence of costimulatory factors, cytokines, or toll-like receptor ligands that can rescue B cells triggered for tolerance induction. Low-affinity autoreactive B cells, B cells that do not encounter self antigen at the immature or transitional stage, and B cells rescued from deletion enter into the naive, mature, immunocompetent B cell repertoire (5).

For these reasons and because autoreactivity is generated de novo by somatic mutation of Ig variable region (V) genes, tolerance mechanisms continue to operate in the periphery. Even antigen-activated B cells can be subject to tolerance mechanisms. IL-6 has been reported to mediate tolerance in B cells subject to chronic antigen exposure (6). Antigen-activated B cells can also be blocked from becoming antibody-secreting plasma cells (7). It is controversial whether receptor editing, or receptor revision as it has been termed in mature cells, is a tolerance mechanism that restricts the autoreactivity generated in an ongoing B cell response.

**Nonstandard abbreviations used:** AID, activation-induced deaminase; APC, allophycocyanin; BCR, B cell receptor; IgH, Ig heavy chain; IL-7R, IL-7 receptor; KLH, keyhole limpet hemocyanin; qPCR, quantitative real-time PCR; Sm, Smith antigen; Tet, tetramer-reactive; V, variable region.

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Results

RAG is induced in postactivation early memory/preplasma B cells following DWEYS-MAP immunization. BALB/c mice immunized with DWEYS-MAP develop T cell–dependent, anti-DNA/antipeptide, cross-reactive antibody responses (15, 18). Using a tetrameric form of this peptide, we are able to detect the tetramer-reactive (Tet+) compartment, which is enriched in DNA-reactive B cells (16, 17). In a previous study, we reported that RAG expression is detectable in a subpopulation of the splenic antigen-reactive compartment within a short time window following immunization with DWEYS-MAP (17). In this study, we sought to further delineate the developmental stage of antigen-reactive RAG-expressing cells and to understand the mechanism and function of receptor editing in an ongoing response.

First, we analyzed spleen sections prepared on day 16 following DWEYS-MAP immunization to determine the location of antigen-reactive and RAG-expressing B cells, at the time we knew them to display maximum RAG expression (17). We used B220 to identify B cell follicles and peanut agglutinin (PNA) to identify GCs. Histologic analysis indicated that there were Tet+ cells within GCs, but RAG did not localize to the GCs (Figure 1A). Instead, staining of spleen sections with antibody to RAG2 and fluorochrome-tagged tetramer demonstrated RAG2 expression in extrafollicular tetramer-binding cells but not in antigen-binding cells in follicles or GCs (Figure 1B).

We next tried to ascertain the differentiation state of antigen-reactive, RAG-expressing B cells. Based on the expression of B220, Tet+ cells can be divided into a major B220lo subset (around 1% of B cells) and a minor B220hi subset (around 0.2% of B cells) (17). Previously, we hypothesized that B220lo cells derive from B220hi cells, as the B220hi subset included a large percentage of cells expressing the GC marker GL-7 and activation markers, and the B220lo subset included a larger percentage of CD138+ plasma cells (17). In order to determine which population expressed RAG, we isolated Tet+B220hi and Tet+B220lo B cells by fluorescence-activated cell sorting (FACS) (Figure 1C) and measured Rag1 and Rag2 mRNA levels by quantitative real-time PCR (qPCR) in these populations. Both Rag1 and Rag2 transcripts were upregulated only in the Tet+B220lo B cell population, and the degree of upregulation was approximately 30% of that seen within GCs, but RAG did not localize to the GCs (Figure 1A). Instead, staining of spleen sections with antibody to RAG2 and fluorochrome-tagged tetramer demonstrated RAG2 expression in antigen-binding cells in extrafollicular loci but not in antigen-binding cells in follicles or GCs (Figure 1B).
in immature BM B cells (Figure 1D). Single-cell PCR analysis showed that approximately 30% of Tet+B220lo B cells expressed RAG2 transcript (data not shown). This was confirmed by histology analysis of spleen sections and was consistent with flow cytometry results reported previously (17).

In T cell–dependent antibody responses, B cells can be activated either to form GCs or to locate in extrafollicular areas and become plasmablasts (19). Because tetramer-binding B cells were detected in both GCs and extrafollicular loci, while RAG+ cells localized only in extrafollicular sites, we asked whether the extrafollicular antigen-reactive RAG+ cells were derived from a GC response or from an extrafollicular response or both. We, therefore, immunized the mice with DWEYS-MAP in CFA on day 0 and boosted with DWEYS-MAP in incomplete Freund adjuvant on day 7. Cells were sorted on day 16 (A–C). (D) Flow cytometry analysis of surface CD80 and CD95 on Tet+B220lo (solid line), Tet+B220hi (dotted line), and Tet-B220lo (line over shaded area) subsets. Mice were immunized with DWEYS-MAP in CFA on day 0 and boosted with DWEYS-MAP in incomplete Freund adjuvant on days 7 and 56. Spleen and BM cells were prepared 4 days after the second boost for analysis. Representative histogram from 4 to 5 mice was shown.

Figure 2
Characterization of antigen-reactive and nonreactive B cells. (A) qPCR analysis of transcripts of μ and γ1 chains in antigen-reactive and nonreactive B cells. (B) Sequence (Seq) analysis of VH and Vk genes expressed by antigen-reactive B cells. Filled circles and triangles represent the number of point mutations in individual VH genes (n = 41) and Vk genes (n = 28) expressed by Tet+B220lo cells. (C) qPCR analysis of Aid, Blimp1, and Xbp1 in antigen-reactive and nonreactive B cells. Data (mean ± SEM) are representative of 3 independent experiments (A and C). ND, not detected. Mice were immunized with DWEYS-MAP in CFA on day 0 and boosted with DWEYS-MAP in incomplete Freund adjuvant on day 7. Cells were sorted on day 16 (A–C). (D) Flow cytometry analysis of surface CD80 and CD95 on Tet+B220lo (solid line), Tet+B220hi (dotted line), and Tet-B220lo (line over shaded area) subsets. Mice were immunized with DWEYS-MAP in CFA on day 0 and boosted with DWEYS-MAP in incomplete Freund adjuvant on days 7 and 56. Spleen and BM cells were prepared 4 days after the second boost for analysis. Representative histogram from 4 to 5 mice was shown.
the Ig V genes expressed by the Tet B220lo B cells were primarily unmutated (data not shown). These data suggest that the Tet B220lo population has undergone GC differentiation, while the Tet B220hi population, which we previously showed included GL-7+ cells, might be in an early stage of the GC response.

Determination of antigen-activated B cells is accompanied by a program of gene expression (21, 22). Activation-induced deaminase (AID) is highly expressed in GC B cells and required for both somatic hypermutation and class switch recombination. By qPCR, we observed that AID was upregulated about 2-fold in Tet B220hi cells compared with non–antigen-specific Tet B220lo B cells (Figure 2C). While AID expression in the Tet B220lo subset was lower than in a population of isolated GC B cells (data not shown), it was substantially higher than in naive Tet B220lo B cells. In contrast, Aif was undetectable in the Tet B220lo population. Blimp1, a transcriptional repressor, and Xbp1, a transcriptional activator, are required for plasma cell differentiation. In contrast to AID, expression of these 2 genes was upregulated to a greater extent in the Tet B220lo compartment than the Tet B220hi compartment, although both Tet subsets show higher expression than naive B cells (Figure 2C). These observations suggest that the Tet B220lo subset has progressed further through a differentiation pathway than the Tet B220hi subset.

We next wanted to determine if the Tet B220lo subset was a memory cell population. Memory B cell development is characterized by the expression of certain surface markers. Among these markers, CD80 and CD95 have been shown to be expressed at a higher level in memory than naive B cells (23). In order to determine whether the newly generated Tet+ cells develop into memory cells, mice were immunized with DWEYS-MAP as described and boosted 8 weeks later. Four days after the boost, we analyzed the antigen-reactive cells and nonreactive B cells by flow cytometry. In the spleen, both CD80 and CD95 were upregulated in the B220lo and B220hi Tet-binding compartments, as compared with non–antigen-reactive naïve B cells (Figure 2D), with expression on Tet B220lo cells higher than on the Tet B220hi subset. Thus, the Tet B220lo population has features of memory B cells. Because antigen-specific memory B cells appear in the BM after immunization (17, 24), we also analyzed the BM Tet-binding cells and observed memory B cell markers on Tet+ cells (Figure 2D). To access memory function in Tet+ cells, we isolated lymphocytes from spleens of immunized mice, either including or excluding Tet+ cells, and transferred them into mice with a mutation in Il7r (MT mice) (25). CD138+ plasma cells were excluded before transfer. When we boosted the recipient mice, a memory response was induced when the Tet+ cells were present but not in the absence of Tet+ cells (Supplemental Figure 2). Tet+ cells were, therefore, capable of differentiating into antibody-secreting cells upon rechallenge with antigen and, therefore, included memory B cells.

In conclusion, the Tet B220lo B cells identified on day 16 after immunization were early memory/preplasma B cells that were isotype switched, hypermutated, and exiting the GC reaction, while the Tet B220hi cells represented an earlier stage of differentiation, perhaps GC cells.

Next, we wanted to confirm that RAG was expressed in mature B cells, because the literature on this topic is quite controversial. We transferred BALB/c naïve spleen cells to SCID mice, waited for 2 weeks for transitional B cells to die or mature, and immunized with DWEYS-MAP or MAP-core. RAG was expressed in the SCID spleens after immunization with the DWEYS-MAP but not in

**Figure 3**
DWEYS-MAP induced RAG expression requires IL-7R signaling. (A) Flow cytometry analysis of IL-7R protein on Tet B220lo (solid line), Tet B220hi (dotted line), and Tet B220lo (line over shaded area) subsets. Splenic cells were prepared on day 16 from DWEYS-MAP–immunized mice. The experiment was repeated twice with 5 mice at each time. The Tet B220lo cells display the highest increase in the level of IL-7R protein. (B) qPCR of Il7r on the specified B cell subsets. Cells from DWEYS-MAP–immunized mice were sorted by flow cytometry on day 16. Data (mean ± SEM) represent 1 of 3 independent experiments. Tet B220lo cells show the greatest increase in Il7r mRNA. (C) Histochemical staining of spleen sections for B220 (red) and RAG2 (green). Mice were immunized with DWEYS-MAP on day 0, boosted on day 7, and treated with anti–IL-7R–blocking antibody or with isotype control on days 8, 11, and 14. Spleens were harvested on day 16. Three mice were included in each group. A minimum of 6 pictures were taken for each spleen. The experiment was repeated twice. Spleens from mice treated with anti–IL-7R antibody showed decreased RAG2 expression (green). Follicles are identified by B220 (red). Original magnification: ×50. (D) qPCR analysis of Rag2 in Tet B220lo cells from DWEYS-MAP–immunized mice treated with anti–IL-7R Ab or with isotype control. The procedure for immunization and administration of antibodies was described in C. Cell sorting was done on day 16, following immunization. Data (mean ± SEM) are representative of 3 independent experiments. Rag2 mRNA is undetectable in mice treated with anti–IL7R antibody. For all these experiments, the mice were immunized with DWEYS-MAP in CFA on day 0 and boosted with DWEYS-MAP in incomplete Freund adjuvant on day 7.
Expression of IL-7R on antigen-reactive B cells is dependent on IL-7R function. It has been reported that expression of RAG cells in DWEYS-MAP–immunized mice was caused by contamination of the transferred cells with immature B cells or pro- or pre-B cells, we isolated naive mature (AA4.1-HSA) lymphocytes from BALB/c mice and adoptively transferred them to RAG2−/− mice. In this experiment, immature B cells were excluded from transfer, and RAG transcript was undetectable in the sorted cells prior to transfer (data not shown). In addition, RAG transcript was not detected in the spleen after splenocytes were transferred to RAG2−/− mice prior to immunization (data not shown).

When we immunized the recipient mice with DWEYS-MAP, we again detected both RAG1 and RAG2 expression in the Tet−B220lo subset (Supplemental Figure 3). Thus, there are no immature B cells exiting from the BM in RAG2−/− mice, the B cell repertoire in the recipient mice was composed exclusively of mature B cells. These data demonstrate that the appearance of RAG+ B cells in the spleen of DWEYS-MAP-immunized mice is not a result of contamination by immature B cells or BM emigrants. Rather, RAG is reinduced in mature immune-competent B cells in an antigen-dependent manner.

**RAG expression and receptor revision in antigen-reactive B cells is dependent on IL-7R function.** It has been reported that expression of RAG proteins, or their functionality, in developing B cells is dependent on IL-7R signaling (26–30). Therefore, we wanted to know whether IL-7− is also involved in the regulation of RAG expression in the spleen of DWEYS-MAP-immunized mice. First, we analyzed the expression of IL-7R in the antigen-reactive B cells of immunized mice. Both flow cytometry for IL-7R protein expression and qPCR for Il7r mRNA on isolated Tet−B220lo and Tet−B220hi B cells showed that IL-7− was most highly upregulated in the Tet−B220lo population (Figure 3, A and B). Naïve Tet−B220hi B cells showed low expression of IL-7R protein or Il7r mRNA. Once we established specific expression of IL-7R on Tet−B220lo B cells, we asked whether IL-7− signaling was critical for RAG expression. Mice were immunized with DWEYS-MAP on day 0 and boosted on day 7. Either anti-IL-7− blocking antibody or isotype control antibody was administered to the mice on days 8, 11, and 14. Histological analysis of spleen sections showed that the expression of RAG2 was substantially inhibited by treatment of the mice with anti-IL-7−-blocking antibody (Figure 3C). qPCR analysis on sorted cells demonstrated an inhibition of RAG2 at the transcriptional level, as we were unable to detect the RAG2 transcript in the Tet−B220lo compartment of anti-IL-7−-treated mice (Figure 3D). Importantly, there was no toxicity associated with the anti-IL-7− antibody, as the Tet−B220lo cells remained positive for expression of IL-7−, even in mice given anti-IL-7− antibody (Supplemental Figure 4). Thus, the anti-IL-7− antibody did not cause the elimination of cells expressing IL-7−. Because IL-7 has been shown to regulate survival and homeostasis of naive and memory T cells (31, 32), we wondered whether blocking the IL-7− pathway might affect helper T cell function and thus impair the GC response. We, therefore, stained the spleen sections from DWEYS-MAP–immunized mice treated with anti-IL-7− or isotype control antibody for GCs and antigen-binding B cells. Anti-IL-7− neither caused an apparent defect in GC formation nor altered the frequency of tetramer-binding cells (data not shown). Furthermore, when the IgH V gene sequences were analyzed, the mutation rate of Tet−B220lo cells was not changed by anti-IL-7− treatment (Supplemental Figure 4), suggesting that there was no defect in the GC response. These observations suggest that the transient blocking of IL-7− did not impair helper T cell function and the GC response, consistent with the observation that IL-7− is downregulated on T cells upon TCR activation (31, 33). Taken together, the reexpression of RAG in the antigen-activated B cells occurs in the context of an adequate T cell response and requires the function of IL-7− on B cells.

Repeated variable joining rearrangement eventually leads to exhaustion of the recombination potential at the κ chain V gene locus and expression of a light chain. Thus, one molecular signature of receptor revision would be an increase in Igκ− cells, and occasionally, coexpression of Igκ and Igλ light chains (34). In our previous study, we showed that λ light chain is upregulated in the Tet−B220lo cells (17), and a significant portion of Tet−B220lo cells are λ/κ double positive in DWEYS-MAP–immunized mice (17). Although λ light chain is detected in the newly activated Tet+ B cells, when we generated antigen-specific hybridomas from immunized mice, we failed to identify any antibody-secreting cell that expresses λ light chain (35). Similarly, we detected no serum λ− antibody binding to DWEYS peptide using ELISA (data not shown). Thus, the expression of λ light chain is due to receptor revision, rather than positive selection, and inhibition of receptor revision should inhibit λ chain expression. We, therefore, analyzed the expression of λ chain in the presence or absence of anti–IL-7− blocking antibody (Figure 3E). qPCR analysis on sorted cells demonstrated an inhibition of RAG2 at the transcriptional level, as we were unable to detect the RAG2 transcript in the Tet+B220lo compartment of anti–IL-7−–treated mice (Figure 3D). Importantly, there was no toxicity associated with the anti–IL-7− antibody, as the Tet+ B220lo cells remained positive for expression of IL-7−, even in mice given anti–IL-7− antibody (Supplemental Figure 4). Thus, the anti–IL-7− antibody did not cause the elimination of cells expressing IL-7−. Because IL-7 has been shown to regulate survival and homeostasis of naive and memory T cells (31, 32), we wondered whether blocking the IL-7− pathway might affect helper T cell function and thus impair the GC response. We, therefore, stained the spleen sections from DWEYS-MAP–immunized mice treated with anti–IL-7− or isotype control antibody for GCs and antigen-binding B cells. Anti–IL-7− neither caused an apparent defect in GC formation nor altered the frequency of tetramer-binding cells (data not shown). Furthermore, when the IgH V gene sequences were analyzed, the mutation rate of Tet+ B220lo cells was not changed by anti–IL-7− treatment (Supplemental Figure 4), suggesting that there was no defect in the GC response. These observations suggest that the transient blocking of IL-7− did not impair helper T cell function and the GC response, consistent with the observation that IL-7− is downregulated on T cells upon TCR activation (31, 33). Taken together, the reexpression of RAG in the antigen-activated B cells occurs in the context of an adequate T cell response and requires the function of IL-7− on B cells.
of antibody to IL-7R. qPCR analysis demonstrated that expression of mRNA of the λ light chain (Igl-V1) in Tet^B220^lo population was markedly decreased in mice treated with anti–IL-7R–blocking antibody (Figure 4A).

To demonstrate that receptor revision was occurring selectively in antigen-activated B cells, we measured the serum levels of secreted IgM and IgG, antibodies using ELISA. IgM levels were not affected by the treatment with the anti–IL-7R antibody (Figure 4B); however, IgG levels were significantly decreased in mice treated with anti–IL-7R antibody (Figure 4C). Since the total serum level of IgG was not altered by the treatment with anti–IL-7R antibody (Supplemental Figure 5), we concluded that the decreased expression of λ light chain is due to inhibition of receptor revision in antigen-specific B cells that are undergoing or have undergone isotype switching.

Receptor revision is a mechanism to regulate an autoantibody response. The observation that RAG protein was reexpressed in the antigen-experienced, early memory/preplasma B cell subpopulation led us to hypothesize that receptor revision may play a role in the regulation of autoreactivity induced by antigen challenge, and the ability of anti–IL-7R–blocking antibody to inhibit RAG expression allowed us to address this question. We immunized BALB/c mice with DWEYS-MAP and administered anti–IL-7R or isotype control antibody as described. The mice were bled at the specified days after immunization. In order to investigate the memory response, these mice were boosted 8 weeks after the primary immunization and bled weekly thereafter. We found that in the primary response, the production of IgM antipeptide and anti–dsDNA antibodies was not affected by anti–IL-7R treatment; titers of anti-DNA IgG, but not antipeptide IgG, were modestly increased in anti–IL-7R–treated mice (Supplemental Figure 6). During the memory response, we observed a greater increase in serum anti-DNA IgG titers in mice treated with anti–IL-7R–blocking antibody (Figure 5A). The IgG memory response to peptide was not changed by anti–IL-7R treatment (Figure 5B).

These results demonstrate that receptor revision attenuates the autoantibody production arising in the course of a response to foreign antigen, and the inhibition of receptor revision leads to increased expression of autoreactivity.

Soluble antigen induces RAG expression and receptor revision. Because RAG is induced in autoreactive, early memory/preplasma B cells but not in the equivalent compartment in mice immunized with the non-self 10-2 peptide (17), we speculated that engagement with antigen, in this case, with dsDNA outside the GC environment, may be essential for RAG induction. In order to test this hypothesis, we adoptively transferred naive splenocytes into the RAG2^−/−^ mice and immunized the recipient mice with DWEYS-MAP 3 weeks later. From day 9 to day 15 following immunization, we treated these mice with active or heat-inactivated DNase I daily, and analyzed the expression of RAG in antigen-reactive cells on day 16. The purpose of the adoptive transfer was to exclude the presence of new BM emigrants, some of which express RAG. Treatment with active DNase I resulted in a reduction of plasma DNA by approximately 50% (data not shown). qPCR analysis showed that the expression of RAG in the Tet^B220^lo compartment from DNase I–treated mice was markedly decreased compared with the control group (Figure 6A). This result suggests that dsDNA in the host is important for RAG induction.

To address definitely whether antigen can actually induce RAG expression and receptor revision during an ongoing immune response, we analyzed 10-2 peptide–immunized mice. Originally identified as a mimotope of phosphorylcholine (36), immunization with 10-2 peptide does not generate an autoreactive B cell response or induce RAG expression by itself (17). BALB/c mice were immunized with 10-2 keyhole limpet hemocyanin (10-2–KLH). On days 13, 14, and 15 following immunization, soluble 10-2–BSA or BSA was administered and splenocytes were examined for RAG induction on day 16. A different protein carrier was used for administration of soluble antigen in order to address specifically the anti–10-2 response.

qPCR analysis of whole spleen cells showed that RAG1 and RAG2 were upregulated by 2- to 3-fold by treatment with 10-2–BSA compared with treatment with BSA alone (Figure 6B). Treatment with 10-2–BSA also resulted in an increase of IgG transcript in antigen-reactive B cells (Figure 6C). Finally, 10-2–BSA administration led to a significant increase of IgG-associated λ light chain compared with BSA treatment, another measure of receptor revision (Figure 6D). λ light chain associated with IgM was not affected by the treatment with 10-2–BSA (Figure 6D). These results indicate that soluble antigen induced receptor revision in antigen-activated B cells.

Receptor revision reduces the serum antibody response. Next we asked whether induction of receptor revision alters the production of 10-2–specific antibody. Because soluble antigen has been shown to induce apoptosis in GC B cells and reduce memory B cell gener-

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**Figure 5**

Receptor revision contributes to tolerance maintenance in peptide-induced autoimmunity. BALB/c mice were immunized with DWEYS-MAP peptide and administered anti–IL-7R antibody or isotype control as described in Figure 3. Eight weeks after the primary immunization, the mice were boosted one more time with DWEYS-MAP in incomplete Freund adjuvant. Anti-dsDNA (A) and antipeptide (B) IgG serum antibodies were measured by ELISA after the boost. Data are the mean ± SEM of 5 mice in each group, representative of 2 independent experiments. The P value was determined by 2-way ANOVA.
Soluble antigen induces RAG and receptor revision in the spleen of immunized mice. (A) Expression of RAG in DWEYS tetramer-binding cells requires the presence of circulating dsDNA in the plasma. BALB/c naive splenocytes were adoptively transferred to Rag2−/− mice. Three weeks later, the recipient mice were immunized with DWEYS-MAP as described in Figure 3. From day 9 through day 15 following immunization, 500 μg active or heat-inactivated bovine DNase I were administered to the mice. Tetramer-reactive cells were sorted on day 16, and Rag1 and Rag2 were analyzed by qPCR. (B) Soluble 10-2–BSA induces RAG in the spleen of mice immunized with 10-2–KLH. (C and D) Receptor revision is induced in antigen-reactive B cells by soluble 10-2–BSA. BALB/c mice were immunized with 10-2–KLH in CFA on day 0 and boosted with 10-2–KLH in incomplete Freund adjuvant on day 7. On days 13, 14, and 15, 1 mg of 10-2–BSA or BSA were administered. qPCR analysis for the mRNA of λ chain (Igl-V1) was done on 10-2–tetramer reactive cells, and nonreactive naive B cells were sorted on day 16 (C). Data (mean ± SEM) are representative of 2 independent experiments. Serum levels of IgG were measured by ELISA (D). Five mice were included in each group. The experiment was repeated twice. **P < 0.01, 2-tailed Student’s t test.
checkpoints have been described. In a study analyzing tolerance induction in human B cells, Tsuji et al. reported that although up to 20% of mature naive B cells express antibodies with self-reactivity, these B cells are excluded from the repertoire during the transition from IgM naive to IgM memory B cells before the onset of somatic hypermutation (41), implying that tolerance induction is operative in the immunocompetent, naive B cell repertoire in humans also.

William et al. reported that 2 checkpoints exist in the periphery to regulate rheumatoid-factor (RF) expressing autoreactive B cells in mice after exposure to antigen (42). While these cells expand and undergo somatic mutation in the autoimmune MRL/lpr background in a non-autoimmune host, they neither clonally expand nor differentiate into antibody-secreting cells. Tolerance was also reported to operate at a preplasma cell stage in anti-Smith antigen (anti-Sm) IgH Tg B cells (43). Anti-Sm B cells are present at a high frequency in normal mouse spleen and BM and express the plasma cell marker CD138. However, these cells do not differentiate into antibody-secreting cells in a non-autoimmune strain. Regulation of anti-Sm B cells occurs before the expression of Blimp1, the transcription repressor required for plasma cell differentiation (43). Although it is also observed that anti-Sm B cells have higher turnover rates than B cells not binding Sm, clonal deletion cannot be the only mechanism for tolerance induction, since a high percentage of anti-Sm B cells are still present in the mice. It was determined that IL-6 secreted by macrophages and dendritic cells helps maintain B cell tolerance in this model (46).

GCs are dynamic microenvironments for B cell differentiation after antigen stimulation. Within GCs, activated B cells rapidly proliferate and undergo somatic hypermutation in their Ig genes. B cells with increased affinity to the eliciting antigen are positively selected and clonally expand. Random V gene mutation will also often give rise to BCRs that are reactive to self antigen (15, 44, 45). In normal situations, these autoreactive B cells are largely prevented from producing autoantibodies and from entering a memory cell compartment (46). Newly formed hapten-specific post-GC B cells were demonstrated to pass through a window of tolerance susceptibility, evidenced by their functional inactivation or deletion following exposure to hapten conjugated to a carrier not recognized by primed T helper cells (47). In vivo studies confirmed a postactivation checkpoint for B cells. During an ongoing primary immune response in mice, injection of soluble antigen induced extensive apoptosis of GC light zone B cells (10, 37, 38). This B cell death was antigen specific.

Debate has raged over the question of receptor revision. RAG is expressed in pro-, pre-, and autoreactive immature B cells in the BM and downregulated in naive mature B cells (48). It has been reported that immunization can induce the expression of RAG proteins in peripheral lymphoid organs (8, 9). However, the developmental stage of the RAG-expressing B cells was not characterized in these studies. Later studies suggested that the RAG+ cells in the spleen of immunized mice were recent emigrants from BM. While their number increased following immunization, they were antigen-independent and were recruited from the BM by adjuvant alone (49, 50). These observations suggested that RAG expression in spleen cells was a function of an increased population of immature B cells. In contrast to this theory, RAG was induced in isolated IgD+ mature B cells by stimulation with LPS and various cytokines in vitro (9, 26). In human peripheral blood, RAG was found to be expressed in a portion of postswitch (IgG) memory B cells (14). In addition, receptor revision of IgH V genes was observed in IgD+ tonsil cells and led to the production of hybrid heavy chain V genes (51). These observations underscore the continuing uncertainty regarding the expression and function of RAG in peripheral B cells. Furthermore, B cells expressing RAG have also been observed outside of GCs in immunized mice (52) and in extrafollicular areas of human tonsil (53). Detection of light chain rearrangement products in RAG-expressing B cells in the periphery clearly suggested that receptor editing or revision may occur in B cells beyond the immature stage (9, 54–56). In the periphery, it was reported that RAG is turned off by BCR cross-linking (33, 57). However, these studies were not performed on B cells that experienced antigen challenge.

To monitor the expression of RAG during B cell development, GFP reporter mice have been generated (58–60). Mice in which GFP was expressed under a RAG2 promoter showed no increase in RAG-expressing B cells following immunization (58). Antigen-specific B cells were not analyzed in these studies, and it would have been difficult to observe an overall change in the number of RAG-expressing B cells if the increase in RAG expression was limited to antigen-specific cells. Immunization of mice having a
fusion gene of RAG2 and GFP replacing the endogenous RAG2 gene locus led to the appearance of GFP in a subset of splenic B cells, approximately 25% of which expressed GL-7, a marker for GC cells, although also a marker of immature B cells (59). Thus, studies in these mice did not provide definitive evidence for or against receptor revision, although it was reported that RAG-expressing B cells were detected in RAG2−/− mice that were adoptively transferred with RAG+/+ BM cells but not with RAG−/− splenocytes (49).

We have previously reported that immunization with a peptide mimotope of dsDNA (DWEYS-MAP) induces a SLE-like serology in the non-autoimmune BALB/c mouse strain (15). The antibody response is T cell dependent (18). In order to investigate the DNA-reactive B cells participating in the response, we developed a methodology using a peptide tetramer to detect antigen-binding B cells in spleens of immunized mice. The tetramer binding subset is enriched for B cells reactive to dsDNA (16). We immunized BALB/c mice harboring the gene for the RAG2:GFP fusion protein with DWEYS-MAP and observed that approximately 20%–50% of antigen-reactive B cells in the spleen express GFP 16 days after antigen exposure (17). Expression of RAG is antigen dependent and is not observed in mice immunized with a control peptide. The adoptive transfer experiment demonstrated that RAG is de novo induced in mature B cells rather than a consequence of contamination with pro-, pre-, or immature B cells. RAG is specifically induced in the Tet−B220lo B cells that express predominantly V gene mutated and isotype switched antibody.

Analysis of gene expression patterns and surface markers in the antigen-reactive compartments and in naive non–antigen-specific B cells suggests that the Tet−B220lo cells are newly generated early memory/preplasma B cells that may derive from the Tet−B220hi cells, consistent with the results obtained by flow cytometry analysis of antigen-activated B cells in our previous report (17) and by other groups (24). These B cells appear to have matured in a GC environment as they display features of a GC-matured response, heavy chain class switching and somatic hypermutation. Furthermore, we detected antigen-specific cells within GCs. Importantly, histological analysis demonstrated that antigen-reactive B cells first appear within GCs on day 8 following immunization. They are not detectable at extrafollicular loci until day 11. We, therefore, believe that the extrafollicular antigen-reactive B cells have matured through a GC response.

RAG expression can be identified in antigen-binding B cells only in extrafollicular loci, and receptor revision occurs only in the Tet−B220lo cells. IL-7R was upregulated in the Tet−B220lo population also, and the expression of RAG required signaling through the IL-7R. Consistent with this observation, the light chain transcripts are suppressed by treatment with the IL-7R–blocking antibody. When receptor editing was suppressed, IgG-associated, but not IgM-associated, light chain expression was significantly decreased, strongly suggesting that receptor revision occurs in antigen-activated mature B cells that are undergoing or have completed isotype switching. We propose that upregulation of IL-7R on antigen-activated autoantibiotic B cells promotes RAG expression and modulates chromatin accessibility for RAG at the light chain locus. IL-7R may also provide a signal to maintain cell viability that allows time for receptor revision to occur.

Furthermore, we showed that receptor revision plays a role in the regulation of an anti-DNA autoantibody response and reduction in accessible DNA leads to a reduction in RAG expression. We confirmed in a second antigen system that soluble antigen can induce receptor revision in antigen-reactive cells and diminish the antigen-specific antibody response. To our knowledge, this is the first direct evidence of receptor revision as a mechanism of tolerance in antigen-activated B cells.

Because the GC response is a source of self-reactive B cells and pathogenic autoantibodies display heavy chain class switching and somatic mutation, understanding tolerance in the antigen-activated B cell and the GC or post-GC B cell is critical to an understanding of many autoimmune diseases. Previous studies have shown that soluble antigen can lead to the deletion of GC B cells or post-GC B cells. We now extend our insight into B cell selection and suggest that antigen-activated B cells that are cross-reactive with self antigen will be positively selected in the GC. After leaving the GC, they, like transitional B cells, remain susceptible to tolerance induction. In the absence of antigen-specific T helper cells or follicular dendritic cells, engagement of BCR with self-antigen, such as dsDNA, may induce reexpression of the recombination machinery and receptor revision, resulting in the replacement of self-reactive BCR. In our studies of antibody response to 10-2–KLH, we determined that soluble antigen can drive receptor revision. It is possible that antigen need not be soluble to induce receptor revision. For example, we demonstrated that DNase treatment eliminated the antigen that provoked receptor revision in the DWEYS-immunized mice. We do not know if DNase treatment reduces soluble antigen or antigen trapped on a membrane. Tolerance-driven receptor revision is not induced within GCs. There are reasons this may be so. First, recent studies indicate that high activity of PI3 kinase negatively regulates the expression of RAG (61, 62). This suggests that RAG may be actively suppressed in GC B cells, since there is an elevation of PI3 kinase activity during B cell activation in the GC response. Second, RAG expression requires a cell-cycle arrest, while GC B cells undergo intensive proliferation.

Just as there was shown to be a temporal window during which soluble antigen can reduce memory B cell formation by inducing apoptosis (36, 63), receptor revision also appears to be restricted in time, beginning at day 12 after immunization, peaking around day 17, and dissipating by day 22 (17). This would allow self antigen to encounter antigen-activated B cells in an environment that is not replete with T cell help and survival signals from follicular dendritic cells.

In the current study, we have identified a peripheral tolerance checkpoint in antigen-activated B cells, which have undergone V gene hypermutation and isotype switching and seem to derive from a GC response. Reinduction of RAG in antigen-activated autoantibiotic early memory B cells requires IL-7R signaling, and the resulting receptor revision contributes to the regulation of autoreactivity. The identification of receptor revision as a tolerance mechanism of antigen-activated B cells will help the pathogenesis of autoimmunity to be understood and provide a potential target for treating autoimmune disease, as it is clear that peripheral tolerance mechanisms are perturbed in autoimmune disease.

**Methods**

*Mice and immunization.* Eight- to ten-week-old female BALB/c and BALB/c RAG2−/− mice were purchased from The Jackson Laboratory or Taconic and housed in specific pathogen-free facilities. Immunization was performed as described (17). In brief, mice were immunized i.p. with 100 μl of a 1:1
emulsion of CFA (Difco), containing 100 μg of DWEYSVWLSN peptide on a branched polylsine backbone (DWEYS-MAP, AnaSpec) or ADGSGGR-DEMQASMS conjugated to KLH (10-2–KLH, AnaSpec). These mice were boosted with 100 μg of DWEYS-MAP or 10-2–KLH in incomplete Freund’s adjuvant (IFA) (Difco) on the date specified in the text (see the legends for Figures 1, 5, and 6). All animal experiments were carried out according to protocols that were reviewed and approved by the Institutional Animal Care and Use Committees of Columbia University and The Feinstein Institute for Medical Research.

Administration of antibody, DNase I, and soluble antigen. For in vivo blocking of IL-7R in mice immunized with DWEYS-MAP, 1 μg of anti-IL-7Rα (A7R34; eBioscience) was injected i.v. on days 8, 11, and 14 following the primary immunization. To degrade plasma dsDNA, 500 μg of bovine DNase I (Sigma-Aldrich) was injected i.v. from day 9 to day 15 following immunization. Control mice received the same amount of heat-inactivated DNase I. In order to examine whether soluble antigen induces RAG expression in antigen-activated B cells, 1 μg of soluble 10-2–BSA or BSA was injected i.v. into 10-2–KLH-immunized mice on days 13, 14, and 15 after the primary challenge. To block IL-7R signaling, 1 μg of anti-IL-7Rα was administered to these mice on days 12 and 14, respectively. Control mice received the same amount of rat IgG2a isotype control antibody at the same time points.

Generation of peptide tetramer. DWEYSVWLSN-streptavidin-allophycocyanin (DWEYSVWLSN-streptavidin-APC) tetramers, DWEYSVWLSN-streptavidin-Allexa Fluor 488 tetramer, and DWEYSVWLSN-streptavidin-Allexa Fluor 350 tetramer were generated as described (16) with minor modifications. Briefly, 1 volume of biotinylated peptide (1 mg/ml) was mixed with 4 volumes of streptavidin–Allexa Fluor 488 or –Allexa Fluor 350 (1 mg/ml; Invitrogen) or with 5 volumes of streptavidin-APC (1 mg/ml; Invitrogen) and incubated at 4°C for a minimum of 3 hours. Before staining, unconjugated peptide was removed from peptide tetramer by gel filtration using a Bio-Gel P-30 column (Bio-Rad). The 10-2 peptide tetramer was generated in the same way as the DWEYS tetramer. Biotinylated peptide was synthesized by AnaSpec.

Reagents and flow cytometry. Spleens were harvested from immunized mice. Single-cell suspensions were prepared as described (17). The following antibodies were used for flow cytometry analysis: peridinin-chlorophyll-protein complex anti-B220 (RA3–6B2; BD Biosciences — Pharmingen), FITC–anti-κ (X36; BD Biosciences — Pharmingen), PE–anti-λ (goat polyclonal; SouthernBiotech), FITC–anti-CD127 (A7R34; eBioscience), PE–anti-CD138 (281-2; BD Biosciences), FITC–anti-CD95 (15A7; eBioscience), FITC–anti-CD127 (A7R34; eBioscience), and PE–anti-CD95 (15A7; eBioscience). PE–anti-CD138 (281-2; BD Biosciences), FITC–anti-CD80 (16-10A1; BD Biosciences — Pharmingen), and PE–anti-CD95 (15A7; eBioscience). DWEYS-APC tetramer was used to detect antigen-binding B cells. DAPI was added before flow cytometry to exclude dead cells. Data were acquired by using LSRII flow cytometry (BD Biosciences) and analyzed by using FlowJo software (Tree Star Inc.).

Cell sorting. To sort the tetramer binding populations, splenocytes were prepared from 4–5 mice at day 16 after immunization. T cells, monocytes, and dendritic cells were depleted as previously reported (17). Staining was performed as described above. Immediately after staining, cells were resuspended in TRIZOL (Invitrogen) and frozen at −140°C until RNA isolation. Sorting was performed on a FACSARia Flow Cytometer (Becton Dickinson).

Histology. Spleens were removed from naive mice or mice immunized with DWEYS-MAP or MAP-core and frozen in Tissue-Tek OCT Compound (Sakura). Cryosections of 5-μm thickness were prepared and mounted on glass slides. Before staining, slides were fixed with ice-cold acetone for 10 minutes and blocked with PBS, containing 2% BSA, 5% goat serum, and 0.2% Triton X-100 (blocking buffer), for 30 minutes at room temperature. Staining was performed with antibodies in blocking buffer for 1 hour at room temperature. Tetramer staining was performed at 4°C overnight. Slides were mounted in Aqua-Poly/Mount (Polysciences Inc.). Fluorescent microscopy was performed on an Axiocam 11 microscope (Carl Zeiss Microimaging). Image acquisition was performed with a Hamamatsu ORCA-ER camera using the Openlab imaging software (Improvision). According to the fluorochrome used for staining, gray images were colored as follows: red for PE or rhodamine, green for Alexa Fluor 488, blue for Alexa Fluor 350. The following antibodies or reagents were used in staining: Alexa Fluor 488 anti-RAG2 (rabbit polyclonal; BD Biosciences — Pharmingen), Alexa Fluor 350 anti-RAG2, PE–anti-B220 (RA3-6B2; BD Biosciences — Pharmingen), DWEYS–Alexa Fluor 488, and rhodamine–peanut agglutinin. Alexa Fluor 488 or 350 anti-RAG2 antibodies were made using Zenon anti-rabbit IgG labeling kits (Invitrogen).

Ig V gene sequence analysis. RNA was isolated from sorted cells by using TRIZOL reagent (Invitrogen). cDNA was prepared using the iScript cDNA Synthesis kit (Bio-Rad). Igx V gene region message was amplified using the degenerative Vκ primer MSκ and CκE as described (64). Since the Tet B220+ population expresses predominantly IgG1, a pool of forward primers covering the first framework region of heavy chain V genes (VHfr1a-j) and a reverse primer at the first domain of Cγ region (65) were used to amplify the Igx V region genes from the Tet B220+ cells. PCR product was separated by agarose gel electrophoresis (Sigma-Aldrich). A single band of proper size was recovered and subjected to TOPO TA cloning (Invitrogen). Plasmid was made from single colonies and subjected to DNA sequencing. The sequences were compared with germline sequences by using the IMGT/V-QUEST (http://imgt.cines.fr). Point mutations between FR1 to CDR 2 regions were counted. Mutations within the first 25 base pairs at the 3’ end of FR1, which is the primer-binding region, were not counted.

Adaptive transfer. Spleens were removed from naive 10- to 12-week-old BALB/c mice. A total of 4 × 10^6 splenocytes or 2 × 10^7 isolated lymphocytes were injected i.v. into RAG2−/− mice. Recipient mice were immunized according to the protocol described.

qPCR. RNA isolation and cDNA preparation were described above. qPCR was performed by using an ABI 7900 (Applied Biosystems) and analyzed using SDS version 2.3 (Applied Biosystems). Applied Biosystems Gene Expression Assays were used, and the reactions were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) in 20 μl final volume. Standard curves were made for each experiment using total spleen and BM cdNA. Relative template concentration was determined from the standard curve by using Cts determined by the SDS software (Applied Biosystems). The RNA polymerase 2a (polr2a) was used as an internal control. All primers used spanned an intron/exon border. Applied Biosystems Primer IDs were as follows: Igκ, Mm01718956_m1; Igγ, custom designed primers; RAG1, Mm01270936_m1; RAG2, Mm00501300_m1; IL-7Rα (CD127), Mm00434295_m1; AID, Mm00507774_m1; Blimp1, Mm00476128; Xbp1, Mm00457359_m1; Igκ, Mm01611614_m1; RNA pol2a, Mm00839493_m1.

Peptide ELISA. EIA/RIA high-binding 96-well plates (Costar) were coated with 50 μl of DWEYS-MAP or 10-2–ovalbumin at 15 μg/ml in PBS and incubated overnight at 4°C. Plates were blocked with 150 μl of 1% BSA in PBS (HyClone) at 37°C for 1 hour. Fifty microliters of diluted sera in 1% BSA were added to the plates for 1 hour at 37°C. Plates were then washed with PBS-Tween, and 50 μl of alkaline phosphatase-conjugated (AP-conjugated) goat anti-mouse IgM or IgG (SouthernBiotech) diluted 1:1000 in 1% BSA/PBS was added to the plates for 1 hour at 37°C. After washing with PBS-Tween, 100 μl of p-nitrophenyl phosphate solution (Sigma-Aldrich) was added and the OD was monitored at 405 nm, using a Titer-Tek Multiscan ELISA reader (Titertek). To determine how much IgM or IgG was bound to the plate, a standard curve was generated with mouse IgM or IgG (Southern-Biotech) on anti-IgM or anti-IgG adsorbed plates.
dsDNA ELISA. Calf-thymus DNA (Calbiochem-Novabiochem) was dissolved in PBS, sonicated, and filtered through a 0.45-μm nitrocellulose Millipore syringe filter (Millipore) to produce dsDNA in 10- to 20-kbp fragments. Fifty microliters of 100 μg/ml dsDNA was used to coat ELISA/RIA high-binding 96-well plates (Costar) overnight at 37°C. The remainder of the assay was performed as described for the peptide ELISA. To determine how much IgM or IgG was bound to the plate, a standard curve was generated, using mouse IgM or IgG (SouthernBiotech) or IgG1- or IgG2a isotype (BioLegend) as the detection antibody. The rest of the assay was performed as described above. A standard curve was generated, using mouse IgM-λ (11E10; SouthernBiotech) or IgG1-λ (A111-3; BD Biosciences—PharMingen) for quantitation of IgM or IgG associated with λ light chain.

Statistics. P values were determined by 2-way ANOVA or Student’s 2-tailed t test as specified in the text. P values of less than 0.05 were considered significant.

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