Published in final edited form as:

Nature. 1998 July 16; 394(6690): 292–295. doi:10.1038/28419.

***V(D)J* recombinase induction in splenic B lymphocytes is inhibited by antigen-receptor signalling**

Marc Hertz^{*,†,§}, Valérie Kouskoff^{*}, Tetsuya Nakamura[‡], and David Nemazee^{*,†}

^{*}National Jewish Medical and Research Center, Division of Basic Sciences, Department of Pediatrics, Denver, Colorado 80206, USA

[†]University of Colorado Health Sciences Center, Department of Immunology, Denver, Colorado 80206, USA

[‡]University of Tokyo, Institute of Medical Science, Department of Infectious Diseases and Applied Immunology, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108, Japan

Abstract

In lymphocytes, DNA recombinations that generate the antigen-receptor genes can sometimes be reinduced in receptor-bearing cells in a process called receptor editing, which modifies the specificity of the receptor for antigen. In immature B lymphocytes, B-cell antigen receptor (BCR) signalling stimulates immune tolerance by receptor editing^{1–5}. More mature splenic B cells can also be induced to undergo *V(D)J* recombination, which generates diversity in the immune system, either by immunization with foreign proteins^{6–9} or by stimulation *in vitro* with interleukin-4 and lipopolysaccharide^{8–10}. Here we show that immune tolerance is unlikely to induce *V(D)J* recombination in mature B cells, because BCR ligation actively inhibits *V(D)J* recombination induced by interleukin-4 and lipopolysaccharide. Furthermore, immunization of immunoglobulin transgenic mice with ligands of varying avidities for the BCR showed that low-avidity antigen could induce strong *V(D)J* recombination, whereas non-binding or high-avidity ligands could not. These data suggest that *V(D)J* recombination induced during the immune response modifies the antigen receptors of B cells with weak, but not strong, reactivity to antigen, potentially rescuing cells with improved receptor affinity and promoting their contribution to the immune response. Thus BCR signalling regulates *V(D)J* recombination in both tolerance and immunity, but in strikingly different ways.

During B-cell development in the bone marrow, immune tolerance is regulated first by receptor editing and later by the induction of apoptosis¹¹. Despite evidence that apoptosis can eliminate autoreactive cells in germinal centres^{12–14}, it has not been excluded that the renewed recombinase expression observed in mature splenic B cells^{6–10,15} could represent a recapitulation of this pattern of tolerance-induced receptor editing in self-reactive B cells created during the process of somatic hypermutation. Alternatively, *V(D)J* recombination may serve as an additional means of somatic diversification in antigen-reactive cells. A more remote possibility is that a subset of immature or pre-B cells present in the spleen is

Correspondence and requests for materials should be addressed to D.N. (nemazee@njc.org).

[§]Present address: M&E Biotech A/S, Kogle Alle 6, DK-2970 Hørsholm, Denmark.

responsible for all of the recombinase activity documented in these previous studies. To distinguish between these possibilities, we induced *V(D)J* recombination in 3–83 $\mu\delta$ BCR transgenic splenic B cells, and tested the effect of BCR ligation on this induction.

We monitored the frequencies of B cells expressing the immunoglobulin λ light (L) chain because the rearrangement of this locus is normally suppressed in 3–83 $\mu\delta$ transgenic mouse B cells and, unlike endogenously encoded immunoglobulin κ L chains, its protein expression can be readily distinguished from the transgenic immunoglobulin. Treatment of isolated B cells with interleukin-4 (IL-4) and lipopolysaccharide (LPS) stimulated an eightfold increase in the percentage of λ^+ B cells relative to the control culture (Fig. 1a; compare media and LPS þ IL-4 panels) but only a twofold increase in B-cell recovery (Fig. 1b). The absolute number of λ^+ B cells recovered was also greatly increased in cultures containing IL-4 and LPS (Fig. 1c). Importantly, the increase in λ^+ B cells induced by IL-4 and LPS, but not the overall increase in B-cell numbers, was suppressed in cultures supplemented with monoclonal anti- κ -chain antibody (Fig. 1b, c) or with anti-CD79b (Ig β)¹⁶, which should stimulate κ and λ L-chain-bearing cells similarly (Fig. 1b, c). The increase in λ^+ B cells in the cultures containing IL-4 and LPS was also significantly suppressed by inclusion of ϕ 31, which is an M13 phage displaying a peptide specifically bound with high avidity by the 3–83 antibody¹⁷ (Fig. 1a, c). In contrast, BCR non-binding phage, ϕ WT, and a phage with 100-fold lower avidity for the BCR, ϕ 11, failed to suppress the increase in λ^+ B cells induced by IL-4 and LPS. The phage concentration used was sufficiently high to result in equivalent and plateau BCR occupancy by phages ϕ 31 and ϕ 11 (data not shown). Thus, the ability to suppress the increase in λ^+ cells that was induced by IL-4 and LPS was correlated with avid binding to the BCR (Fig. 1c). Because both anti-BCR monoclonal antibodies and phage ϕ 31 could suppress the response, it is unlikely that the suppression required interaction with Fc receptors on B cells. Treatment with IL-4 and LPS also increased λ^+ B-cell numbers in cultures of cells from non-transgenic mice. Once again, this response could be inhibited with anti-BCR antibodies (data not shown), indicating that these results were not dependent on the use of cells from BCR transgenic mice.

To determine whether preferential proliferation of λ^+ or κ^+ B cells contributed to the observed changes in the percentage of λ^+ cells in cultures containing IL-4 and LPS, we monitored the cell divisions by prelabelling cells with the fluorescent dye carboxyfluorescein diacetate succinimidylester (CFSE) and assessing fluorescence after 48 h of treatment *in vitro*. With each division, CFSE-labelled cells lose approximately half of their fluorescence¹⁸. Although the overall proliferation was increased in all samples treated with LPS alone or in combination with IL-4, the extent of proliferation was similar in cultures containing IL-4 and LPS, regardless of the presence of BCR ligands (Figs 1b and 2a). In addition, BCR ligation did not accelerate the rate of apoptosis in the treated samples (data not shown). Furthermore, the proliferation of λ^+ splenic B cells in these cultures was no greater than that of the λ^- B cells and was not increased by treatment with LPS and IL-4, indicating that the increase induced by LPS and IL-4 in λ^+ B cells was not due to a preferential outgrowth of λ^+ B cells over κ^+ B cells; instead, it suggested that new λ -chain gene rearrangements occurred (Fig. 2b).

We tested the predictions that stimuli inducing λ expression increase $V(D)J$ recombinase activity, and that BCR ligation suppresses this response. We found that transgenic B cells treated with LPS and IL-4 had increased levels of the recombinase activator gene *RAG-2* mRNA, as expected from previous work^{8–10}, and found that this response was suppressed by BCR ligation (data not shown). Similarly, treatment with LPS and IL-4 induced the formation of λ gene excision product (Fig. 3, compare lanes 1, 7), which was suppressed by BCR ligation (Fig. 3, lanes 8, 9, 11). In contrast, BCR ligation did not disrupt the ability of treatment with LPS and IL-4 to stimulate sterile Ig γ 1 gene transcription (data not shown). Collectively, these data indicate that new immunoglobulin gene rearrangements are induced in splenic B cells treated with LPS and IL-4 and that, unlike in immature bone-marrow B cells, BCR ligation does not stimulate, but rather inhibits, these gene rearrangements.

To test *in vivo* the possible role of BCR signalling in the $V(D)J$ recombinant response, we immunized 3–83 $\mu\delta$ transgenic mice with recombinant phage displaying peptides that bound with varying avidities to the 3–83 BCR¹⁷ (Fig. 4). We found that these mice do not respond to control phage lacking 3–83-reactive peptide, probably because of the low precursor frequency of reactive B cells, but they respond well to phage expressing 3–83-reactive peptide inserts and begin to form germinal centres by 4–5 days (not shown). The frequency of splenic B cells expressing endogenous λ -chains was increased by immunization with the low-avidity phage ϕ 11 (compare Fig. 4a and d), but not by the non-binding phage ϕ WT (Fig. 4b) or the high-avidity phage ϕ 31 (Fig. 4c). This increase in λ^+ B cells in response to ϕ 11 was probably not a result of outgrowth as a consequence of their improved binding to the phage because the 3–83 H-chain paired with a λ -chain has no detectable affinity for the ϕ WT, ϕ 31 or ϕ 11 phage, as measured by enzyme-linked immunosorbent assay (ELISA) (data not shown). Both the frequency and absolute numbers of λ^+ B cells were consistently increased in mice immunized with the low-avidity phage ϕ 11 (Fig. 4e, f). The increase in λ^+ B cells was the result of new $V(D)J$ recombination, as indicated by analysis of the gene-rearrangement DNA excision products in the spleens of immunized mice. λ excision products were rare in the spleens of unimmunized 3–83 $\mu\delta$ transgenic mice (Fig. 4g, lane 4) and were not substantially increased by immunization with control or high-avidity phage (Fig. 4g, lanes 1 and 2, respectively). In contrast, mice immunized with the low-avidity phage had high levels of λ -gene excision products, indicative of newly created λ gene rearrangements (Fig. 4g, lane 3). It is unlikely that this response is the result of phage stimulating receptor editing directly in the bone marrows of the immunized mice because, if this were the case, phage ϕ 31 should have shown a comparable or better recombinase response. These results are consistent with the idea that B cells with a weak affinity for antigen are able to take up antigen and to initiate an interaction between helper T cell and B cell, but, because of poor signalling of antigen through their BCRs, cannot suppress the effects of T-cell-derived recombinase-inducing signals, such as CD40L and IL-4. In contrast, B cells with a higher affinity for antigen can be protected from the induction of a recombinase response by stronger BCR-mediated signals.

These data support three important conclusions about $V(D)J$ recombination in peripheral B cells. First, they confirm recent reports documenting the ability of splenic B cells to re-express $V(D)J$ recombinase and to undergo secondary immunoglobulin gene

rearrangements^{6–10,15}. Second, the ability of BCR ligation to dampen the expression and activity of the recombinase in splenic B cells indicates that cells undergoing *V(D)J* recombination express surface immunoglobulin and do not represent pre-B-cell contamination; this was not clear in earlier reports. Finally, the ability of BCR ligation to diminish the recombinase activity in splenic B cells suggests that *V(D)J* recombination in peripheral B cells is not tolerance-driven, as previously suggested^{6,8–10}. Although *RAG* gene expression and recombinase activity in germinal-centre centrocytes^{7,9,10} could suggest that self-reactive B cells created during the process of somatic hypermutation may initiate receptor editing, our data and other less direct evidence⁷ make this possibility seem unlikely. The stimuli required to induce the peripheral B-cell recombinase response seem to be T-cell-derived signals, and BCR signalling in this context actively suppresses *V(D)J* recombination in splenic B cells, whereas BCR signalling in immature surface immunoglobulin-bearing bone-marrow B cells stimulates receptor editing^{3,11}. These striking differences suggest that *V(D)J* recombination has different roles in the contexts of tolerance and immunity.

During the antibody response, competition among B cells for antigen binding favours the expansion or survival of rare cells that have a high affinity for antigen¹⁹. Inhibition of receptor revision by the antigen would potentially provide a way of stimulating the expansion of high-affinity clones while allowing lower-affinity cells to improve their affinities. Our phage-immunization results suggest that some BCR signal is required for recruitment to the immune response, but that a strong signal may prevent subsequent receptor revision, at least while antigen quantities last, whereas a weak signal allows receptor revision. It should be remembered, however, that the putative *in vivo* diversification response that we have studied occurs early in, or perhaps before, the germinal-centre response, suggesting that, during the T-cell-dependent response, receptor revision may be initiated before somatic hypermutation.

Within the bone marrow, immature B cells undergo receptor editing to solve a self-tolerance problem, whereas in the periphery receptor revision may contribute to the important processes of intraclonal competition and diversification by stimulating rearrangements in B cells that fail to bind antigen through their BCR. According to this model, receptor revision, like antigen-induced somatic mutation, is likely to create, and potentially select, receptors with affinity to self-tissue. Thus, unlike receptor editing in immature bone-marrow B cells, receptor revision does not solve a self-tolerance problem, but rather seems likely to create one. This situation is reminiscent of T-cell positive selection in the thymus^{20,21}, in which selection for receptors is associated with termination of *V(D)J* recombination, but also with increased risk of autoreactivity, perhaps explaining why both processes are associated with subsequent negative selection by apoptosis^{12–14,22}.

Methods

Mice and immunizations

We generated 3–83 $\mu\delta$ transgenic mice²³ by breeding homozygous 3–83 $\mu\delta$ transgenic males with B10.D2nSn/J (Jackson Laboratories) females. All mice were bred and maintained in the animal care barrier facility at the National Jewish Medical and Research Center. Mice 8–

12 weeks old were immunized intraperitoneally with 25 µg of recombinant M13 phage emulsified in complete Freund's adjuvant (Gibco) and analysed 4–5 days later.

Culture conditions

Splenic B cells were prepared and cultured as described⁸. Briefly, spleens were removed aseptically from mice 8–12 weeks old, cells were dispersed between sterile frosted-glass slides in 10 ml ice-cold sterile HBSS (Gibco) and filtered through sterile cotton to remove debris. Red blood cells were lysed with buffered ammonium chloride. B cells were isolated by anti-Thy-1 and complement depletion of T cells followed by Percoll (Pharmacia) density separation (ρ 1:062) as described²⁴. Purified splenic B cells (10^6 per ml) were cultured in IMDM (Gibco) supplemented with 10% fetal calf serum, 1% sodium pyruvate, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 2 mM L-glutamine and 50 µM 2-β-mercaptoethanol, and maintained at 37 °C with 7% CO₂. Control anti-Ars idotype (5 Ci) (from L. Wysocki), monoclonal anti-κ, 331812 (from T. Rolink), anti-CD79b (Ig-β)¹⁶, HM79 and recombinant M13 phage were added to a final concentration of 10 µg ml⁻¹, IL-4 (Pharmingen) was added to a final concentration of 500 U ml⁻¹, and LPS (Gibco) was added to a final concentration of 20 µg ml⁻¹.

Flow cytometric analysis

Cells were analysed by two- or three-colour FACS on a FACS Calibur flow cytometer (Becton Dickinson). Anti-B220-PE (Caltag) was used at a dilution of 1:250; goat anti-mouse λ L-chain-FITC (Caltag) was used at a dilution of 1:1,000; anti-B220-biotin (RA3-3A1, ATCC) was used at a dilution of 1:200; anti-mouse λ-1,2-biotin (Pharmingen) was used at a dilution of 1:200; and anti-IgM^a-biotin (Pharmingen) was used at a dilution of 1:200. All biotin-conjugated antibodies were revealed with avidin-tricolor (Caltag) at a dilution of 1:50.

Rearrangement polymerase chain reaction (PCR) assay

To generate DNA containing templates for PCR, 2×10^5 cells were incubated in 200 µl lysis buffer at 55 °C overnight, and amplified by PCR for α-actin or λ L-chain excision products^{1,3}. This assay amplifies and detects the excised DNA episomes generated as the product of Vλ1-to-Jλ1 rearrangements.

Acknowledgements

We thank L. Wysocki and T. Rolink for reagents and discussion, and members of D.N.'s lab for critical review of the manuscript and discussion. V. Kouskoff was supported by an EMBO fellowship. This work was supported by grants from the NIH and the Arthritis Foundation.

References

1. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med.* 1993; 177:1009–1020. [PubMed: 8459201]
2. Gay D, Saunders T, Camper S, Weigert M. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med.* 1993; 177:999–1008. [PubMed: 8459227]
3. Hertz M, Nemazee D. BCR ligation induces receptor editing in IgM⁺IgD⁻ bone marrow B cells in vitro. *Immunity*. 1997; 6:429–436.

4. Chen C, Prak EL, Weigert M. Editing disease-associated autoantibodies. *Immunity*. 1997; 6:97–105. [PubMed: 9052841]
5. Pelanda R, et al. Receptor editing in a transgenic mouse model: site, efficiency and role in B cell tolerance and antibody diversity. *Immunity*. 1997; 7:765–775. [PubMed: 9430222]
6. Han S, Zheng B, Schatz DG, Spanopoulou E, Kelsoe G. Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. *Science*. 1996; 274:2094–2097. [PubMed: 8953043]
7. Han S, et al. V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science*. 1997; 278:301–305. [PubMed: 9323211]
8. Hikida M, et al. Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science*. 1996; 274:2092–2094. [PubMed: 8953042]
9. Hikida M, Ohmori H. Rearrangement of λ light chain genes in mature B cells in vitro and in vivo. Function of reexpressed recombination-activating gene (RAG) products. *J. Exp. Med.* 1998; 187:795–799. [PubMed: 9480989]
10. Papavasiliou F, et al. V(D)J recombination in mature B cells: a mechanism for altering antibody responses. *Science*. 1997; 278:298–301. [PubMed: 9323210]
11. Melamed D, Benschop RJ, Cambier JC, Nemazee D. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell*. 1998; 92:173–182. [PubMed: 9458042]
12. Pulendran B, Kannourakis G, Nouri S, Smith KG, Nossal GJ. Soluble antigen can cause enhanced apoptosis of germinal-centre B cells. *Nature*. 1995; 375:331–334. [PubMed: 7753199]
13. Shokat K, Goodnow CC. Antigen-induced B-cell death and elimination during germinal-centre immune responses. *Nature*. 1995; 375:334–338. [PubMed: 7753200]
14. Han S, Zheng B, Dal PJ, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 1995; 182:1635–1644. [PubMed: 7500008]
15. Hikida M, Mori M, Kawabata T, Takai T, Ohmori H. Characterization of B cells expressing recombination activating genes in germinal centers of immunized mouse lymph nodes. *J. Immunol.* 1997; 158:2509–2512. [PubMed: 9058779]
16. Nagata K, et al. The Ig α /Ig β heterodimer on μ -negative proB cells is competent for transducing signals to induce early B cell differentiation. *Immunity*. 1997; 7:559–570. [PubMed: 9354476]
17. Kouskoff V, et al. Partial agonist ligands of the B cell receptor induce differential lymphokine production and partial tyrosine phosphorylation (submitted to *J. Exp. Med.*).
18. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods*. 1994; 171:131–137. [PubMed: 8176234]
19. Kelsoe G. Life and death in germinal centers (redux). *Immunity*. 1996; 4:107–111. [PubMed: 8624801]
20. Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Ann. Rev. Immunol.* 1995; 13:93–126. [PubMed: 7612239]
21. Fink PJ, Bevan MJ. Positive selection of thymocytes. *Adv. Immunol.* 1995; 59:99–133. [PubMed: 7484464]
22. Marrack P, Hugo P, McCormack J, Kappler J. Death and T cells. *Immun. Rev.* 1993; 133:119–129. [PubMed: 7901146]
23. Russell DM, et al. Peripheral deletion of self-reactive B cells. *Nature*. 1991; 354:308–311. [PubMed: 1956380]
24. Pleiman CM, Hertz WM, Cambier JC. Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science*. 1994; 263:1609–1612. [PubMed: 8128248]

Figure 1.

IL-4/LPS treatment of isolated 3–83 μ 8 transgenic splenic B cells induces an increased percentage and number of λ^+ B cells; this effect can be inhibited by BCR ligation. Data show the effects of various culture conditions on the percentage and absolute number of λ^+ cells after 48 h culture. **a**, Two-colour FACS analysis of treated cultures. The upper number in each histogram shows the percentage, and the lower number the absolute number, of λ^+ /B220 $^+$ cells per $\sim 10,000$ gated B cells. **b**, Total viable B220 $^+$ cells. **c**, Total viable λ^+ /B220 $^+$ cells. Data are mean $\pm$ s.d. of 5 experiments.

Figure 2.

CFSE cell-division analysis of cultured B220⁺ and λ⁺ 3–83 μδ transgenic splenic B cells. B cells were stained with CFSE before culture and stimulated for 48 h as indicated in Fig. 1. Broken vertical lines mark successive cell divisions. **a**, FACS analysis of CFSE label in B220⁺ cells at 48 h. **b**, Analysis of CFSE label among gated λ⁺/B220⁺ lymphocytes. The number in the top left corner of each histogram indicates the median fluorescence intensity as an indication of cell division.

Figure 3.

BCR ligation blocks secondary L-chain gene rearrangements induced in purified 3–83 $\mu\delta$ transgenic splenic B cells by IL-4/LPS treatment *in vitro*. Autoradiographs of PCR products revealed by Southern blot analysis of λ L-chain excision products (λ XP, top) and α -actin control gene (Actin, bottom) in cells treated as indicated in Fig.1.

Figure 4.

Immunization of 3-83 $\mu\delta$ transgenic mice with phage bearing a low-avidity 3-83 peptide ligand induces secondary immunoglobulin L-chain gene rearrangements *in vivo*. **a-d**, Flow cytometry analysis of splenic lymphocytes 4-5 days after immunization with: **a**, PBS; **b**, control M13 phage, ϕ WT; **c**, phage ϕ 31 expressing high-avidity 3-83 peptide ligand; **d**, phage ϕ 11 expressing low-avidity peptide ligand. Splenic cells (2×10^6) were stained with anti-B220-PE, anti- λ -FITC and anti-IgM^a-biotin. **e**, Percent λ^+ B cells. **f**, Number of λ^+ B cells per 20,000 cells analysed within the lymphocyte gate. Light bars represent total lymphocytes and dark bars represent a subset of IgM^a transgenic H-chain-expressing lymphocytes. In **e** and **f**, data are mean $\pm$ s.d. **g**, Increased endogenous λ gene rearrangement excision products in splenic B cells from mice immunized with low-avidity peptide ligand. PCR analysis of λ gene rearrangement excision product (XP, top) and α -actin control gene (Actin, bottom) using cell lysate templates made from 3-83 $\mu\delta$ transgenic splenic B cells 4 days after immunization with: control M13 phage (lane 1); phage ϕ 31 high-avidity peptide ligand (lane 2); phage ϕ 11 low-avidity peptide ligand (lane 3); unimmunized 3-83 $\mu\delta$ H-2^d non-deleting (ND) spleen to show untreated background levels of λ excision products (lane 4); and unimmunized 3-83 $\mu\delta$ H-2^b central deleting (CD) spleen as a positive control to show the extent of λ excision products detected in mice undergoing extensive secondary immunoglobulin L-chain gene rearrangements¹ (lane 5).