

Neoteny in Lymphocytes: *Rag1* and *Rag2* Expression in Germinal Center B Cells

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(GAPDH). These PCR cycles were confirmed to be in the exponential phase of the amplification. PCR products were electrophoresed on 7.5% polyacrylamide gel and visualized by Southern (DNA) blotting using ³²P-labeled probes, the Dde I-Dde I 163–base pair (bp) internal fragment of RAG-1 cDNA, the Pst I-Hinf I 124-bp internal fragment of RAG-2 cDNA, and the entire coding region of GAPDH cDNA. Hybridized filters were exposed to Fuji imaging plate (Fuji Film) for 2 days and visualized by a Bioimaging Analyzer, BAS 1000 (Fuji Film). RAG-1 and RAG-2 cDNAs were given by D. G. Schatz (Yale University, New Haven, CT).

19. Mouse B cells were prepared by treating spleen cells from male C3H/HeN mice (8 to 10 weeks of age, Japan Charles River) with 1/1000-diluted mAb to Thy 1.2 mAb (SeroTec), followed by incubation with low-toxic rabbit complement (Cederlane) as described [K. Haruna *et al.*, *Cell. Immunol.* **151**, 52 (1993)]. The B cells (3×10^6 cells per milliliter) were cultured with LPS (20 μ g/ml) from *Escherichia coli* 055 B5 (Sigma) and mouse recombinant IL-4 (500 U/ml; PeproTech) in 1 ml of RPMI-1640 medium containing 10% fetal bovine serum, 10 μ M 2-mercaptoethanol, penicillin G (100 U/ml), and streptomycin (50 μ g/ml). In some cases, mAb to mouse CD40 (1 μ g/ml; rat mAb LB429 presented by N. Sakaguchi, Kumamoto University, Japan) or F(ab')₂ fragment of goat antibody to mouse μ heavy chain (10 μ g/ml; Cosmo Bio) plus 1 mM 8-mercaptopguanosine (Sigma) was used as a stimulus. Because it was confirmed that RAG expression peaked on day 2 of the culture and declined thereafter, all cultures were carried out for 2 days. In *in vivo* experiments, mice were immunized with 20 μ g of TNP-KLH and 0.45 mg of alum in each hind footpad. Inguinal or popliteal LN cells from three mice were pooled on day 0, 6, and 8 postimmunization and assessed for the expression of RAG-1 and RAG-2 mRNA (18).
20. Culture plates of 100 mm diameter were coated with 100 μ g/ml of mAb to mouse IgD (Bioss). Sixty million mouse spleen cells that had been depleted of T cells and erythrocytes were placed in the plate and incubated for 1 hour at room temperature. Then the plate was gently washed with phosphate-buffered saline four times to remove nonadherent cells. It was confirmed by flow cytometric analysis (FACScan) that adherent cells recovered from the plate were more than 99% positive for slgD and B220.
21. Monoclonal antibodies to mouse RAG-1 (G109-256.2, mouse IgG2b) and RAG-2 (G110-461, mouse IgG2b) were obtained from Pharmingen. A myeloma-derived murine IgG2b (MOPC 195) was used as an irrelevant negative control (ICN Biomedicals). These mAbs were biotinylated under the same conditions, using a biotinylation kit (American Qualex). Staining of RAG proteins in thymocytes or cultured B cells was carried out as described (7), with some modifications. Briefly, the cultured cells were fixed on glass slides in methanol-acetone (1:1) for 5 min, rehydrated in phosphate-buffered saline, and preblocked for 1 hour with TBST [10 mM tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 1% bovine serum albumin (BSA) and MOPC 195 (50 μ g/ml). The slides were then incubated in TBST containing 1% BSA and one of each biotinylated mAb (5 μ g/ml) for 1 hour at room temperature. Slides were then washed three times in TBST and reacted for 1 hour with rhodaminated avidin (2 μ g/ml; Sigma) in TBST containing 1% BSA. For the immunofluorescent staining of LN sections, 6- μ m-thick cryosections mounted on slides were allowed to air dry for 15 min and were fixed in ice-cold acetone for 10 min. After rehydration and pre-blocking as described above, the sections were treated with biotinylated anti-RAG-1 (5 μ g/ml) for 1 hour, followed by double-staining with rhodaminated avidin (2 μ g/ml) and FITC-PNA (4 μ g/ml; Seikagaku Kogyo) for 40 min. All reagents were diluted in TBST containing 1% BSA. After washing with TBST, samples were finally mounted with low-fluorescent glycerol and cover slip protection, and were observed with a Zeiss fluorescence microscope.
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The products of the *Rag1* and *Rag2* genes drive genomic V(D)J rearrangements that assemble functional immunoglobulin and T cell antigen receptor genes. Expression of the *Rag* genes has been thought to be limited to developmentally immature lymphocyte populations that in normal adult animals are primarily restricted to the bone marrow and thymus. Abundant RAG1 and RAG2 protein and messenger RNA was detected in the activated B cells that populate murine splenic and Peyer's patch germinal centers. Germinal center B cells thus share fundamental characteristics of immature lymphocytes, raising the possibility that antigen-dependent secondary V(D)J rearrangements modify the peripheral antibody repertoire.

Periodic expression of the recombination-activating genes *Rag1* and *Rag2* controls the assembly of immunoglobulin (Ig) genes and defines the principal stages of B lymphopoiesis in the bone marrow (1). Transcription of the *Rag* genes ends with the expression of competent Ig on the surface of immature B cells, precluding further V(D)J recombination in the mature lymphocyte pool (2). However, we and others have found that lymphocytes in germinal centers (GCs) exhibit features of immature T and B cells, including the expression of membrane markers typically present on developing lymphocytes (3) and exquisite sensitivity to activation-induced apoptosis that is independent of the Fas molecule (4). Perhaps most remarkable is the similar spectrum of nucleotide exchanges introduced during antigen-driven V(D)J hypermutation in murine GCs and by the developmentally regulated generation of point mutations in the Ig genes of B cells in ileal Peyer's patches (PP) of fetal lambs (5).

Germinal centers are sites of antigen- and T cell-dependent cellular reactions that develop in secondary lymphoid tissues. Germinal centers are necessary for immunological memory in the B cell compartment (6, 7) and are the site of V(D)J hypermutation and selection that is required for affinity maturation of antibody responses (8). Two populations of B lymphocytes, the mitotically active Ig⁻ centroblasts and the nondividing Ig⁺ centrocytes, make up the majority of GC cells; centrocytes arise from centroblasts, and in turn, some centrocytes reenter the proliferating pool (3, 9). Evidence suggests that the centrocyte population is subject to selective apoptosis (4). Splenic GCs first appear 4 to 5 days after primary immunization and may be identified by their distinctive ability to bind peanut agglutinin (PNA⁺) and the monoclonal antibody GL-7 (GL-7⁺) (3). The GC reaction is transient, peaking by day 12 of the response and waning after 3 weeks (9). In contrast, GCs are constitutively present in murine PPs, being chronically stimulated by food antigens and the gut flora (10).

To determine if the immature character of GC B cells extended to the level of *Rag* expression, we used affinity-purified antibodies specific for active RAG1 and RAG2 proteins (11) to label histologic sections of spleen and PP from immunized and normal mice (12). Mature GCs, those present in spleen 16 days after immunization (Fig. 1) or in the PPs of unimmunized mice (Fig. 2), contain PNA⁺, GL-7⁺ B cells that express substantial amounts of immunoreactive RAG1 and RAG2 protein. The distribution of labeled cells coincided with the distribution of B7-2 expression, suggesting that RAG proteins are predominantly expressed in the centrocytes of the GC light zone (7). Virtually identical staining patterns for immunoreactive RAG1 were achieved with rabbit IgG specific for the NH₂-terminal residues of RAG1 and a murine monoclonal antibody that binds to the COOH-terminal region of RAG1 (13). Histologic demonstration of RAG2 was more difficult, even in sections of thymus, a site of active V(D)J recombination and high RAG expression (1). Two rabbit antibodies were used to localize RAG2 protein; one, made against amino acids 70 through 516 of murine RAG2, gave equivocal labeling, whereas the other (antibody 435), specific for a 20-amino acid stretch of RAG2 (13), adequately labeled both GCs (Figs. 1C and 2B) and cortical thymocytes.

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The presence of immunoreactive RAG1 and RAG2 in GC B cells was further supported by using the reverse transcriptase-dependent polymerase chain reaction (RT-PCR) assay to detect the presence of RAG1, RAG2, and hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA (14) in small numbers (5×10^3 to 2×10^4 cells) of GC (GL-7⁺B220⁺) and follicular (GL-7⁻B220⁺) B cells purified by fluorescence-activated cell sorting (15) from spleens of immunized mice. Comparable numbers of immature, CD4⁺CD8⁺ (double-positive) thymocytes were similarly prepared to serve as controls for the RT-PCR assay. RAG1 and RAG2 message was readily detected in double-positive thymocytes and in as few as 5×10^3 GC B cells (Fig. 3). Re-analysis of sorted GC B lymphocytes indicated enrichment of GL-7⁺B220⁺ cells to only ~35% compared with ≥96% for follicular B cells and double-positive thymocytes. In contrast, neither RAG1 nor RAG2 message could be detected in even larger numbers (2×10^4 cells) of follicular B cells or lipopolysaccharide-activated B cell blasts (Fig. 3). Approximately equivalent amounts of HPRT mRNA were present in all cell cohorts, indicating generally equivalent recoveries of intact RNA (Fig. 3). Sequence analysis of RAG RT-PCR products from GC B cells confirmed these to be RAG1 and RAG2.

GC B cells have been proposed to represent

a distinct lineage of B lymphocytes (16). Do the B cells that migrate into nascent GCs already carry the RAG proteins or is their expression induced by the GC microenvironment? In contrast to mature GCs, only about half of newly developed splenic GCs contain B cells that express detectable amounts of RAG proteins. The number of RAG⁺ centrocytes in GCs and the intensity of their labeling increases during the GC reaction, suggesting that events within the GC microenvironment up-regulate RAG1 and RAG2 expression (Fig. 4). This pattern of expression mirrors the onset of $\mu \rightarrow \gamma 1$ Ig class switching and the accumulation of point mutations in the Ig heavy chain genes of GC B cells (17). However, Ig class switching simultaneously occurs in RAG⁻ B cells located within extrafollicular foci of antibody-secreting cells (18), and immunization with pneumococcal vaccine, a type-II T cell-independent antigen (19), induces RAG1⁺ GCs in the absence of significant levels of V(D)J hypermutation (20). These observations imply that RAG proteins are not necessary for Ig class switching nor sufficient for V(D)J hypermutation. Indeed, extensively mutated Ig light chain transgenes have been recovered from Ig transgenic *Rag1*^{null} mice reconstituted with specific T helper cells and antigen (21). By day 19 after immunization, a fraction of B cells within GCs had lost the ability to bind PNA or GL-7 but remained

positive for immunoreactive RAG1 and RAG2. RAG2 protein also persists in newly generated B cells beyond the cessation of transcription (2), suggesting that the V(D)J recombinase may be briefly present in B cells that have exited GCs.

Expression of RAG1 and RAG2 in GCs reveals the GC microenvironment as a site that supports a population of peripheral B cells profoundly similar to pre-B cells in the bone marrow. The many phenotypic characters shared by developing B cells and those in GCs (3) extend to reactivation of the V(D)J recombinase. The antigen-dependent GCs of mice may represent evolutionary homologs of gut-associated tissues that drive developmentally regulated diver-

Fig. 1. Immunohistological staining of a single GC in serial splenic sections. Adjacent, 6- μ m sections (A to E) through the spleen of a C57BL/6 mouse immunized with NP-CGG 16 days earlier were stained with (A) peanut agglutinin (PNA) (red), (B) rabbit antibody to RAG1 (anti-RAG1) (blue), (C) rabbit anti-RAG2 (blue), (D) normal rabbit Ig (blue), and (E) GL-7 antibody (blue). (F) The GC structure and adjacent splenic architecture are diagrammatically illustrated. GL-7 and PNA label centroblasts and centrocytes to define the location of the GC through the intervening sections; note that the GC column rotates clockwise as it follows the periarteriolar lymphoid sheath and central arteriole through the splenic white pulp. LZ, light zone; DZ, dark zone; ca, central arteriole; pals, periarteriolar lymphoid sheath, the splenic T cell zone. Magnification $\times 90$.

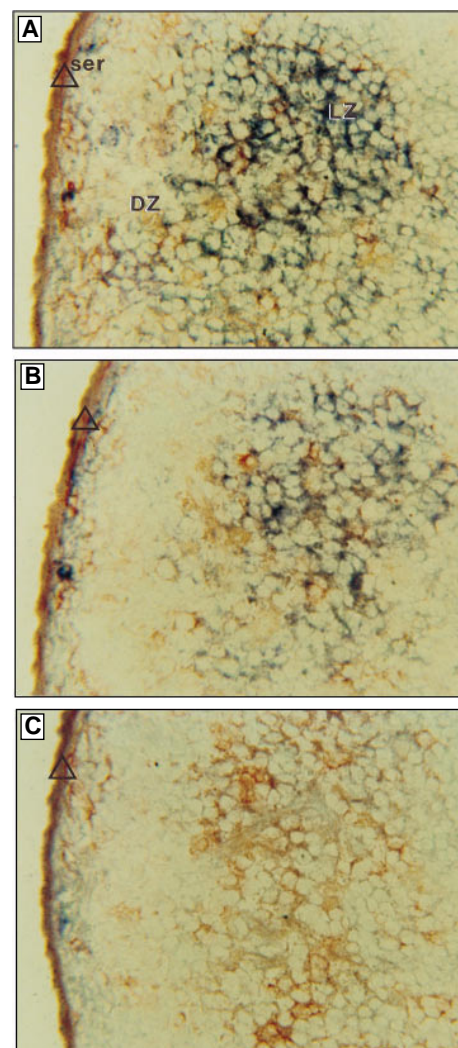
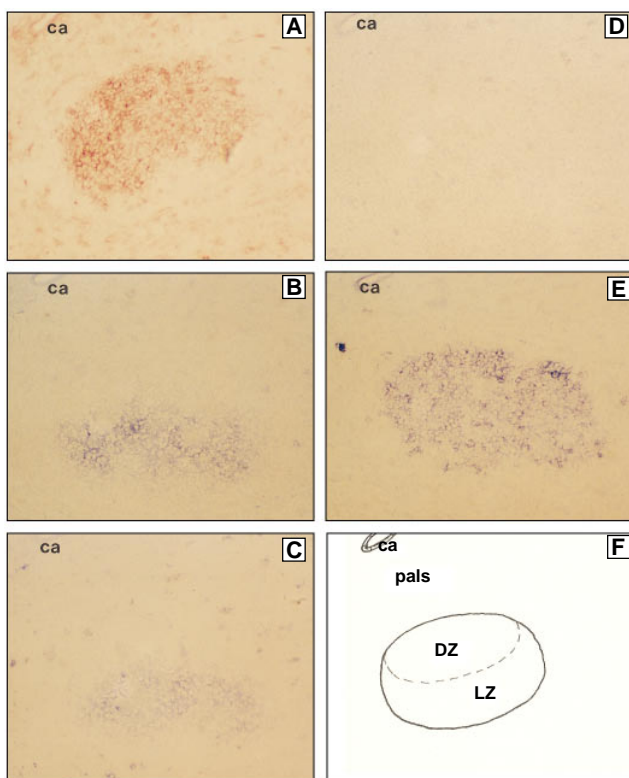


Fig. 2. Expression of RAG1 and RAG2 in PP germinal centers. Adjacent sections of PP from naive C57BL/6 mice were labeled with (A) rabbit anti-RAG1 (blue) and PNA-HRP (red), (B) anti-RAG2 (blue) and PNA-HRP (red), and (C) normal rabbit Ig (blue) plus PNA-HRP, as described (12). Doubly labeled cells appear black. LZ, light zone; DZ, dark zone; ser, serosal surface of the PP. Magnification $\times 208$.

sification of Ig after rearrangement in other vertebrate species (3, 5, 22). However, in mice these properties are present in both the intestinal PPs and splenic GCs (Figs. 1 and 2). Induction of an immature-like state in GC lymphocytes may reflect a mechanism to remove autoreactive cells that arise by mutation (4, 9). The physiologic state that permits this selective apoptosis may coordinately reactivate RAG1 and RAG2.

The availability of V(D)J recombinase in GC B cells also suggests several possibilities for the diversification of Ig genes in centrocytes. RAG1 and RAG2 could mediate secondary V(D)J rearrangements leading to light chain replacement or the introduction of new V_H gene segments by means of cryptic recombination signals present near their 3' termini (23). Light chain receptor editing is commonly observed in autoreactive immature B cells driven to initiate apoptosis by Ig engagement—a scenario not unlike the fate of self-reactive centrocytes (4). In fact, a significant fraction of human B cells that express the λ light chain carry productively rearranged κ light chain genes that have been inactivated by somatic mutation (24). Other evidence consistent with light chain replacement comes from genetic analysis of follicular lymphomas, tumors that exhibit many characteristics of GC lymphocytes including V(D)J hypermutation (25). Sklar *et al.* (26) have reported one tumor composed of two clonal lymphomas related by a common Ig heavy chain rearrangement but distinct by virtue of dissimilar light chain genes. It may be

significant also that the t(14;18) chromosomal translocation present in most follicular lymphomas is thought to arise as an error of V(D)J recombination (27). Documentation of secondary V(D)J rearrangements in GCs would constitute a striking exception to one of immunology's funda-

mental tenets: that antigen does not elicit the formation of novel receptors.

Note added in proof: Messenger RNA specific for the $\lambda 5$ component of the pre-B cell receptor complex (2) can be readily detected by a specific RT-PCR assay in as few as 5×10^5 GC (GL-7⁺B220⁺) B cells. In contrast, $\lambda 5$ message was not found in larger numbers (5×10^4) of follicular B cells (GL-7⁻B220⁺). These findings further substantiate the immature character of B lymphocytes in GCs.

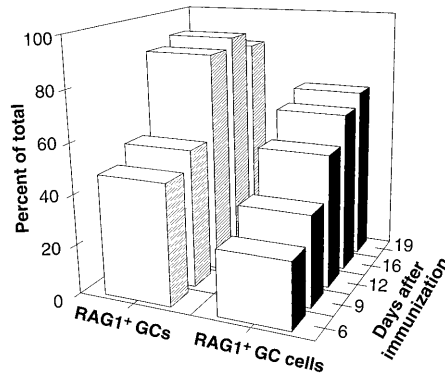
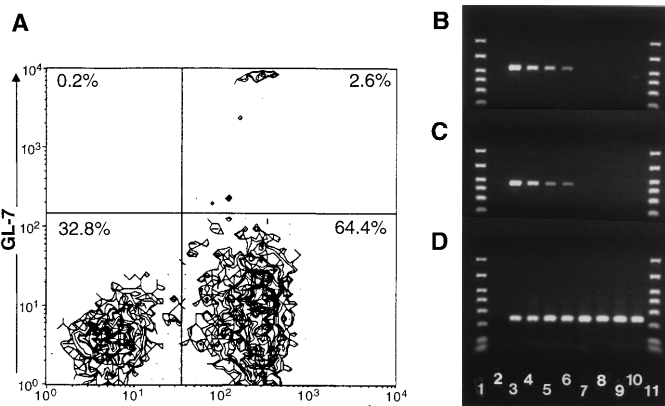


Fig. 4. Kinetics of RAG expression in GCs. C57BL/6 mice were immunized with NP-CGG (72); spleens were removed at days 6, 9, 12, 16, and 19 after immunization and frozen for immunohistology. Sections were co-stained with rabbit anti-RAG1 and PNA. The fraction (percent of total PNA⁺ GCs; shaded bars) of PNA⁺ GCs that also expressed immunoreactive RAG1 ($\geq 5\%$ RAG⁺ cells per GC) were determined by the inspection of 474 PNA⁺ GCs. The frequencies (percent of total PNA⁺ cells; black bars) of RAG1⁺ cells in RAG⁺ GCs were established by direct enumeration in a randomly selected subset (118 GCs) of this population. Data for days 6 through 16 are means from two to three mice at each time point; the value for day 19 represents a single mouse.

Fig. 3. Flow cytometric analysis and RT-PCR assay to evaluate RAG expression in GC B cells. Single-cell suspensions of splenocytes were prepared from three C57BL/6 mice 16 days after immunization with NP-CGG for the isolation of GC and follicular B cells by flow cytometry. (A) Total splenic cells were stained as described (15). Percentages of gated cells for each population are indicated. B220 versus GL-7 defines GC B cells (GL-7⁺B220⁺) and follicular B cells (GL-7⁻B220⁺). (B to D) Subpopulations including GL-7⁺B220⁺ and GL-7⁻B220⁺ spleen cells and CD4⁺CD8⁺ thymocytes were isolated (range: 5×10^3 to 2×10^4 cells). Cellular RNA was reverse transcribed with primers specific for *Rag1*, *Rag2*, and *HPRT* (14). The cDNA was then amplified with 5' and nested 3' primers (15) specific for (B) *Rag1*, (C) *Rag2*, and (D) *HPRT*, yielding products of 546, 472, and 249 bp, respectively. PCR products were detected by staining with ethidium bromide. Lane 1, molecular size markers; lane 2, no reverse transcriptase control (1×10^4 CD4⁺CD8⁺ thymocytes); lane 3, 1×10^4 CD4⁺CD8⁺ thymocytes; lane 4, 5×10^3 CD4⁺CD8⁺ thymocytes; lane 5, 1×10^4 GL-7⁺B220⁺ spleen cells; lane 6, 5×10^3 GL-7⁺B220⁺ spleen cells; lane 7, 2×10^4 GL-7⁻B220⁺ spleen cells; lane 8, 1×10^4 GL-7⁻B220⁺ spleen cells; lane 9, 2×10^4 lipopolysaccharide (LPS)-stimulated (48 hours) spleen cells; lane 10, 1×10^4 LPS-stimulated (48 hours) spleen cells; lane 11, molecular size markers.



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12. C57BL/6 mice (female, 6 to 8 weeks old) were immunized intraperitoneally with 100 μ g of alum-precipitated (4-hydroxy-3-nitrophenyl)acetyl coupled to chicken γ -globulin (NP-CGG). Serial, 6- μ m-thick sections of spleens from immune mice and PPs from naive mice were stained with a murine monoclonal antibody specific for RAG1 or affinity-purified rabbit antibodies to RAG1 or RAG2 (13). Normal rabbit Ig (Sigma) was used as a negative control. The antibodies were incubated with tissue sections at 4°C overnight, followed by incubation with goat F(ab)₂ antibody to rabbit Ig, conjugated to biotin (Southern Biotechnology, Birmingham, AL). Bound, biotinylated antibody was then detected with streptavidin-alkaline phosphatase (SA-AP). The same or adjacent sections were stained with PNA coupled to horseradish peroxidase (HRP) (E-Y Laboratories, San Mateo, CA) or biotinylated GL-7 antibody followed by

- staining with SA-HRP or SA-AP (Southern Biotechnology) to detect the GCs (19). Bound HRP and AP activities were visualized with 3-aminoethyl carbazole (3-AEC) (red stain) and fast blue BB (blue strain), respectively. Commercially obtained rabbit IgG specific for terminal deoxynucleotidyl transferase or rat Ig failed to label splenic and PP GCs.
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 14. Two microliters of cDNA from each 20- μ l sample reverse transcribed from extracted RNA was amplified in RT-PCR assays which used the following primer pairs: *Rag1*, 5'-CCAAGCTGCAGACATTCTAGCACTC-3', 5'-CTGGATCCGGAAAATCCTGGCAATG-3'; *Rag2*, 5'-CACATCCACAAGCAGGAAGTACAC-3', 5'-GGTTCAGGGACATCTCCTACTAAG-3'; and HPRT, 5'-GCTGGTGAAGGACCTCT-3', 5'-CAGGACTAGAACCCTGC-3'. Taq polymerase (Gibco, Gaithersburg, MD) was used in all amplifications. PCR was carried out as follows: for *Rag1*—at 94°C for 2 min followed by eight cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, and 22 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; for *Rag2*—at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; for HPRT—at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min. The amplified products (6 μ l) were loaded onto a 1% agarose gel and visualized with ethidium bromide. To ensure that RT-PCR products were not derived from contaminating genomic DNA, the primer pairs for *Rag1* and *Rag2* each span an intron and primers for HPRT span two introns. The molecular sizes of the PCR products were determined by comparison to standards of 50, 100, 200, 300, 400, 500, 700, and 1000 base pairs (bp) (Marker XI, Boehringer-Mannheim).
 15. Splenic cells obtained from C57BL/6 mice immunized with NP-CGG were stained with fluorescein isothiocyanate-conjugated antibody to mouse B220 (PharMingen, San Diego, CA) and GL-7-biotin followed by staining with SA coupled to phycoerythrin (PE). GL7⁺B220⁺ and GL-7⁻B220⁺ cell populations were sorted into Trizol reagent (Gibco). Some cells were sorted into cold medium for re-analysis. Routinely, GL-7⁺B220⁺ cells were 33 to 55% pure, whereas GL-7⁻B220⁺ populations were \geq 96% pure. Similarly, CD4⁺CD8⁺ thymocytes were also sorted to serve as positive controls; these double-positive thymocytes were \sim 99% pure. Total RNA recovered from Trizol digests of the different sorted populations was reverse-transcribed with SuperScript (Gibco) with the following specific primers according to the manufacturer's instructions: *Rag1*, 5'-CTGGGAAGTAGACCTGAC-3'; *Rag2*, 5'-CCCATGCTTTCCCTCGACT-3'; and HPRT, 5'-GACG-CAGCACTGACATTTTC-3'.
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Enhancement of Class II-Restricted T cell Responses by Costimulatory NK Receptors for Class I MHC Proteins

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An important feature of the human immune system is the ability of T cells to respond to small quantities of antigen. Class II major histocompatibility complex (MHC)-restricted T cells that expressed a costimulatory natural killer (NK) cell receptor for class I MHC proteins were cloned. In the presence of low doses of superantigen, the proliferative response of these T cell clones was three- to ninefold greater when the T cells were costimulated by way of the NK receptor. Thus, the action of costimulatory NK receptors on T cells may play a significant role in initiating and sustaining immune responses.

T cells recognize antigens through contacts made between the T cell receptor (TCR) and peptides presented in association with specific MHC proteins on an antigen-presenting cell (APC). However, the T lymphocyte response is also shaped by many other interactions between cell-surface molecules on T cells and APCs, as well as by the action of cytokines (1). Although no distinct "antigen receptor" analogous to the TCR has been found on NK cells, NK cell-mediated lysis can be inhibited by NK receptors that also bind to class I MHC proteins (2, 3). In particular, lysis by NK1 and NK2 cells is inhibited by target cells expressing human leukocyte antigen (HLA)-Cw2, -Cw4, -Cw5, or -Cw6 and HLA-Cw1, -Cw3, -Cw7, or -Cw8, respectively (4, 5). Also, lysis by NK1⁺ NK3 cells is inhibited by target cells expressing an HLA-B allele containing the Bw4 epitope at residues 77 to 83 (6). Such inhibition is initiated by the recruitment of protein tyrosine phosphatases on the cytoplasmic tail of the NK receptor (7). T cells share with NK cells a common lineage and

many phenotypic markers (8) including NK inhibitory receptors. Indeed, \sim 28% of cytotoxic T cells express p58 NK inhibitory receptors (9), and the p70 NK inhibitory receptor, NKB1, is expressed on 0.2 to 15% of T cells (10). The action of these NK inhibitory receptors can affect T cell function because NKB1⁺ T cell clones that can kill superantigen-coated target cells cannot kill the same target cells transfected with class I MHC alleles expressing the Bw4 epitope (10).

Recently, an isoform of NK inhibitory receptors was described. These p50 NK receptors share similar extracellular sequences with the p58 receptors but have altered transmembrane regions, including the addition of a charged lysine residue, and truncated cytoplasmic tails (3, 11), so that they lack the YXXL sequences (L, Leu; Y, Tyr; X, any amino acid) to which protein tyrosine phosphatases can bind. Such short-tailed NK receptors do not mediate inhibition of NK cell-mediated lysis but instead activate or coactivate NK clones (11). To investigate the possible effect of NK activating receptors on T cell function, we studied T cell clones isolated as by-products in NK cell cloning (12).

Two of these clones, TANK-1 and TANK-9, were prepared from a donor whose HLA type is HLA-A1, -A2, -B7,

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Neoteny in Lymphocytes: *Rag1* and *Rag2* Expression in Germinal Center B Cells

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