genes. Results from four representative CTL clones (two $CD4^+TCR\alpha\beta^+$ and two $CD8^+TCR\alpha\beta^+$ T cell clones) are shown in Fig. 3. The protection from lysis is conferred by expression of HLA class I on the target cells because addition to the cytotoxicity assay of $F(ab')_2$ fragments of mAb to HLA class I restored killing of the SEB-coated transfectants (Fig. 3). Superantigen-induced cytolysis of 721.221 target cells was inhibited by mAb to CD3, showing involvement of the TCR-CD3 complex (*22*). Thus, CTL clones have, in addition to NKB1, a number of functionally similar receptors for HLA class I molecules that inhibit the cell-mediated cytotoxicity induced by the interaction between TCR on the effector T cells and SEB bound to MHC class II on the target APC. Although we used SEB in our experiments, similar results were obtained with other superantigens such as SEA and TSST-1 (*22*).

Our results indicate that many T cell clones may express inhibitory receptors that recognize polymorphic HLA class I molecules. Engagement of these receptors by their HLA class I ligands on potential target cells substantially limits the cytotoxicity induced by bacterial superantigens. These MHC class I receptors may also play a more general role in the regulation of T cell responses against alloantigens and conventional peptide antigens. In particular, inhibitory signals generated through these HLA class I receptors may prevent destructive autoimmunity against normal tissues in circumstances where T cells are activated during an inflammatory immune response (*23*, *24*).

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Cloning of Immunoglobulin-Superfamily Members Associated with HLA-C and HLA-B Recognition by Human Natural Killer Cells

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Cytotoxicity by natural killer (NK) cells is inhibited by major histocompatibility complex (MHC) class I molecules on target cells. This inhibition may be mediated by NK receptors with different MHC specificities. A family of four NK-specific complementary DNAs (cDNAs), designated NKATs (NK-associated transcripts), was identified that encoded related transmembrane proteins, characterized by an extracellular region with two or three immunoglobulin-superfamily domains and by a cytoplasmic domain with an unusual antigen receptor activation motif (ARAM). The distribution of these cDNAs was clonotypic and correlated with NK cell inhibition by particular class I alleles. Thus, NKAT cDNAs may encode receptors for class I molecules on NK cells.

Cytotoxicity by NK cells is controlled by activating and inhibitory receptors (*1*). Receptors that activate NK cells, such as the Fc γ receptor III (FcγRIII) and NKR-P1, belong to the immunoglobulin (FcγRIII) or to the C-type lectin (NKR-P1) superfamilies and trigger NK cytotoxicity when bound to an immunoglobulin Fc region or to a carbohydrate ligand on target cells, respectively (*2*). Receptors that inhibit NK cells turn off NK cytotoxicity when engaged with an MHC class I molecule on target cell (*3*). The only NK inhibitory receptor characterized, Ly-49, belongs to the C-type lectin superfamily (*4*). In humans, NK cell clones derived from the same individual are heterogeneous in their recognition of class I molecules, suggesting that there may be multiple NK inhibitory receptors with different MHC specificities that are clonotypically distributed (*5*). NK clones exist that are inhibited by Asn^{77}-Lys^{80} human leukocyte antigen–C (HLA-C) alleles (NK1-specific clones), by Ser^{77}-Asn^{80} HLA-C alleles (NK2-specific clones), and by Ile^{80} HLA-B alleles (NK3-specific clones) (*6*). Monoclonal antibodies for human NK inhibitory receptors have been reported, but the genes have not yet been identified (*7*–*9*).

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To clone these receptors, we amplified human NK cell mRNAs by reverse transcription–polymerase chain reaction (RT-PCR) using several sets of degenerate oligonucleotides derived from highly conserved regions shared by known molecules of the immunoglobulin-superfamily (Ig-SF) and C-type lectin superfamily. We searched for amplified cDNAs preferentially displayed by NK cells as compared to B, T, and myeloid cells (*10*).

Among several amplified fragments, we selected a ~240–base pair (bp) cDNA amplified from the NK cell clone 3D2 with one set of Ig-SF–specific degenerate primers (*11*). This fragment hybridized in Northern (RNA) blot analysis to a ~1.9-kb transcript and to a less abundant transcript of ~1.7 kb, which were detected in peripheral blood lymphocytes (PBLs), in the NK cell line NK3.3 (*12*), and in the tumor cell line SKW3 (*13*) (Fig. 1). No hybridization was observed with other tissues or with the B, T, and myeloid cell lines tested, suggesting that this cDNA is selectively expressed on NK cells (*14*).

A full-length cDNA sequence containing the 240-bp fragment was obtained by amplification of cDNA 5′ and 3′ ends (*15*). The resulting 1605-bp cDNA sequence, termed NKAT1 (NK-associated transcript 1), contains a single open reading frame that is predicted to encode a transmembrane protein of 348 amino acids with a

molecular size of 38.5 kD (Fig. 2). The amino acid sequence begins with a typical hydrophobic signal peptide of 21 amino acids followed by an extracellular region of 224 amino acids, with five potential N-linked glycosylation sites. The putative transmembrane domain is represented by a 19-residue stretch of hydrophobic amino acids followed by an 84–amino acid long cytoplasmic domain.

Comparison of the predicted amino acid sequence of NKAT1 with the protein sequence databases revealed only a ~30% identity with the extracellular regions of human Fc α receptor (FcαR) and murine cell surface antigen gp49, which are members of the Ig-SF (*16*). The NKAT1 extracellular region is composed of two C2-type Ig-SF domains, each with two characteristic cysteines 48 to 50 residues apart and flanked by conserved residues (Val-X-Leu-X-Cys and Leu/His-X-Gly-X-Tyr-X-Cys, respectively, where X is an amino acid). The cytoplasmic region contains two Tyr-X-X-Leu pairs (antigen receptor activation motif or ARAM) spaced by 26 amino acids, rather than six to eight as observed in other hematopoietic antigen receptors (*17*). These results indicated that NKAT1 is a member of the Ig-SF.

Southern (DNA) blot analysis of human genomic DNA revealed several hybridizing bands for each restriction digest, suggesting that a family of genes closely related to NKAT1 may exist (Fig. 3). Genomic DNA analysis of human-hamster hybrid cell lines, each with a different partial complement of human chromosomes, showed hybridizing bands only in samples containing human chromosome 19, revealing that the NKAT gene family maps to human chromosome 19.

To search for closely related molecules expressed on different clones, cDNAs from NK clones derived from the same individual were amplified by RT-PCR with primers based on NKAT1 sequence, cloned, and sequenced (*18*). Distinct but related cDNA sequences, termed NKAT2, NKAT3, and NKAT4, were detected in some of the clones. NKAT2 encodes a transmembrane molecule with two C2-type Ig-SF domains (Fig. 2), which has 92% amino acid identity with NKAT1. NKAT3 and NKAT4 cDNAs also encode transmembrane proteins related to NKAT1, but their extracel-

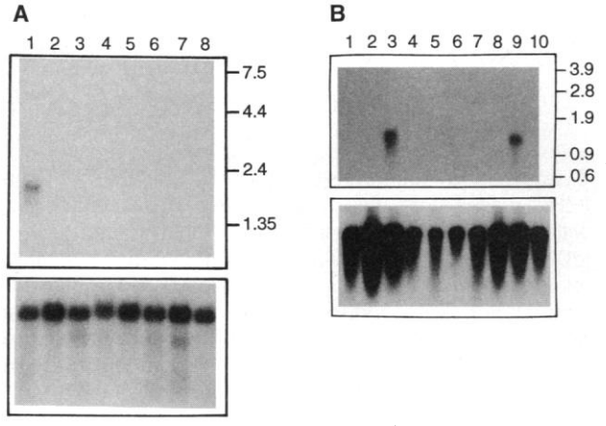

Fig. 1. Expression of the 240-bp cDNA fragment amplified from the NK clone 3D2 in human tissues and hematopoietic cells. (**A**) Lanes 1 to 8: PBLs, colon, small intestine, ovary, testis, prostate, thymus, and spleen. (**B**) Lanes 1 to 10: U937 (monocytic cell line), HL-60 (promyelocytic cell line), SKW3 (leukemia cell line), γδ T cell clone, $CD8^+$ T cell clone, $CD4^+$ T cell clone, Jurkat (T cell), SAVC (EBV-transformed B cell line), NK3.3 (NK cell line), and YT (tumor cell line). Bottom panels, actin controls. Molecular size standards are indicated on the right side (in kilobases).

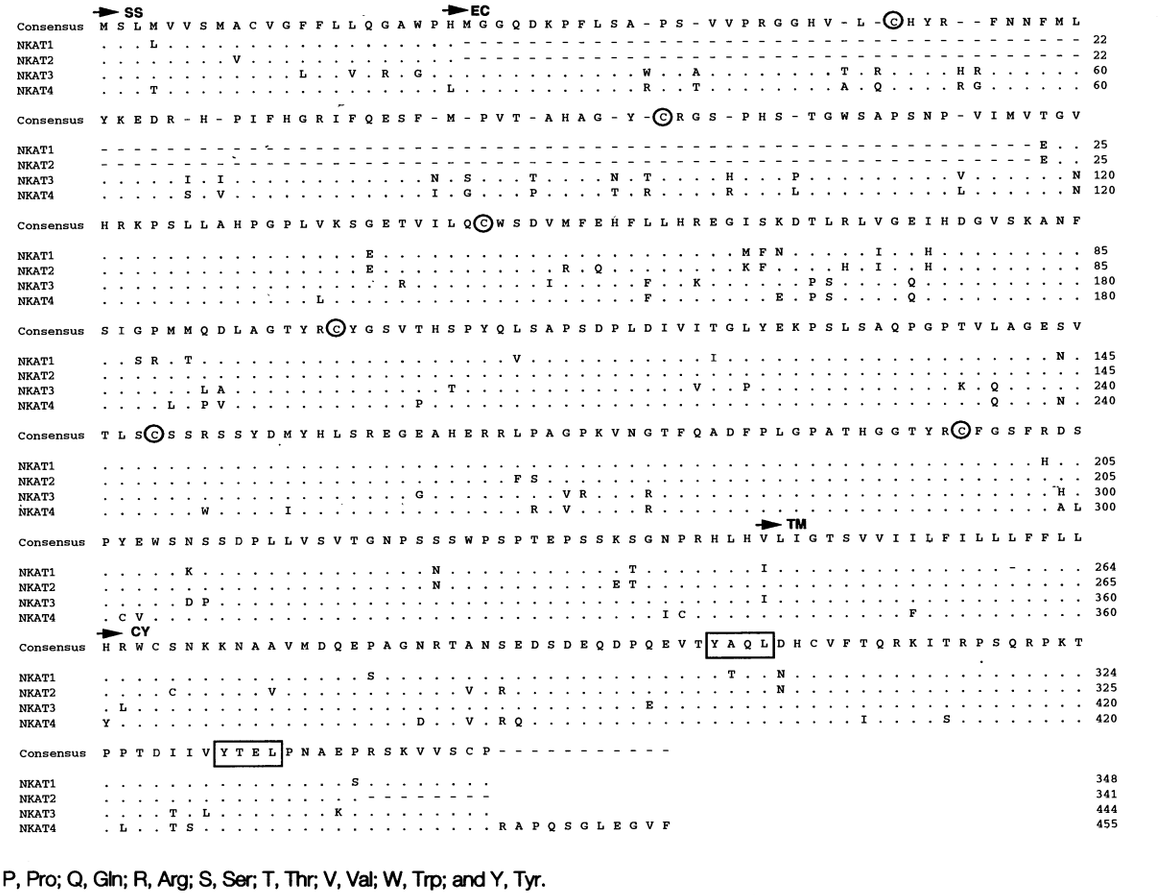

Fig. 2. Alignment of NKAT1, NKAT2, NKAT3, and NKAT4 amino acid sequences. Gaps (dashes) were introduced to maximize homologies. Amino acids identical to the consensus are indicated by dots. Horizontal arrows denote the beginning of the predicted domains. Conserved cysteines involved in potential disulfide bonds in the extracellular domains are circled; tyrosine-leucine pairs of the ARAM are boxed. Amino acid residues are numbered on the right, beginning with the putative initiator methionine. SS, signal peptide; EC, extracellular; TM, transmembrane; CY, cytoplasmic. cDNA sequences have been deposited in GenBank with the following accession numbers: NKAT1, L41267; NKAT2, L41268; NKAT3, L41269; and NKAT4, L41270. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

lular domains consist of three Ig-SF domains instead of two. The predicted NKAT3 and NKAT4 proteins consist of 444 and 455 amino acids with a molecular size of 49.1 and 50.2 kD, respectively (Fig. 2). An alignment of NKAT cDNAs revealed an identity of ~80% within the NKAT group, with divergent amino acids interspersed throughout the extracellular, transmembrane, and cytoplasmic domains, rather than clustered (*19*).

NK cell inhibition by class I molecules is heterogeneous, because different NK clones display specificities for distinct HLA molecules (*6*). To test whether the expression of particular NKAT genes is related to NK specificities for class I alleles, we amplified a cDNA fragment shared by all NKAT genes by RT-PCR from 45 NK cell clones derived from the same donor, and the amplified products were hybridized with oligonucleotide probes specific for each of the NKAT genes (Fig. 4) (*20*). NK clones showed different expression patterns, with most clones expressing multiple genes. NKAT2 was the most widely expressed (34/45), then NKAT4 (29/45), NKAT3 (23/45), and NKAT1 (13/45). More importantly, the clonotypic expression of these genes correlated with the susceptibility of NK cell clones to inhibition by specific class I molecules. All NK1-specific clones (inhibited by Asn^{77}-Lys^{80} HLA-C alleles) expressed NKAT1 (13/45), whereas this gene was not expressed in clones that lack this specificity. NK3-specific clones (inhibited by Ile^{80} HLA-B alleles) expressed either NKAT3 (13/45) or NKAT4 (19/45) or both (10/45), whereas clones that lack NK3 specificity expressed neither. Finally, NK2-specific clones (inhibited by Ser^{77}-Asn^{80} HLA-C alleles) expressed NKAT2 (12/45). However, this gene was also expressed on clones that lack NK2 specificity. This may be explained by the presence of unknown NKAT genes, which do not correlate with NK2 specificity, but cross-hybridize with NKAT2. Alternatively, as suggested (*7*), NK2 specificity may be determined by the cooperation of two molecules, only one of which is encoded by NKAT2.

The p58 molecules have been proposed as candidate receptors for HLA-C (*7, 8*). Two types of p58 molecules have been defined serologically: one, recognized by monoclonal antibodies (mAbs) EB6 and HP3E4; another, recognized by mAb GL183. All the NK1-specific cell clones (inhibited by Asn^{77}-Lys^{80} HLA-C alleles) used in our study expressed NKAT1 and stained positive for HP3E4. More importantly, an HP3E4-negative T cell line became HP3E4-positive after transfection with the NKAT1 gene (*21*) (Fig. 5). Thus, NKAT1 encodes the p58 molecules recognized by mAb HP3E4. NKAT2 resembles NKAT1 in size and amino acid sequence, but is expressed in NK clones with different HLA-C specificity. Thus, NKAT2 may encode for the other p58 molecule, recognized by mAb GL183. A 70-kD glycoprotein recognized by antibody DX9, designated NKB1, has been proposed as a candidate receptor for some HLA-Bw4 alleles (*9*). NKAT3 or NKAT4 (or both) may encode NKB1, because they are expressed in NK cell clones that are inhibited by Bw4 alleles and the predicted size of the encoded protein is close to that of the deglycosylated NKB1 protein (~50 kD).

Our results provide evidence that NK cells express Ig-SF molecules, characterized by heterogeneity and clonotypic distribution, that may be receptors for class I molecules. Interestingly, NKAT-encoded molecules are structurally different from Ly-49, a C-type lectin that is also a receptor for class I molecules in mouse NK cells. Whereas Ly-49 may interact with carbohydrate determinants of class I molecules, NKAT receptors may recognize class I–bound peptides (*22*) or solvent-exposed class I epitopes.

The availability of NKAT cDNAs will make it possible to investigate the mechanisms by which NK cytotoxicity is turned off by MHC recognition. It is possible that NK receptors may behave either as activating or inhibitory receptors depending on their affinity for the MHC class I–peptide complex (*22, 23*). Alternatively, tyrosine phosphorylation of the receptor on the atypical ARAM motif may recruit an SH2-containing protein that blocks NK cell activation, similar to what has been proposed for the FcγRIIB (*24*).

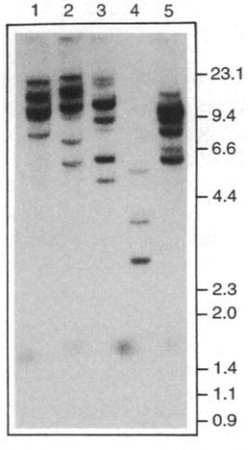

Fig. 3. DNA blot analysis. NKAT1 cDNA hybridized to several restriction fragments of human genomic DNA under conditions of high stringency. Restriction digests were with Eco RI, Hind III, Bam HI, Pst I, and Bgl II (lanes 1 to 5). Molecular size standards are indicated on the right (in kilobases). Human DNA was extracted from PBLs by standard protocol and digested with restriction enzymes. DNA fragments were separated by electrophoresis in a 0.7% agarose gel, transferred to a nylon membrane (Hybond N^+, Amersham) by capillary blot in 0.4% NaOH, and hybridized with NKAT1 probe. Hybridization, washings, and exposure were as described (*14*).

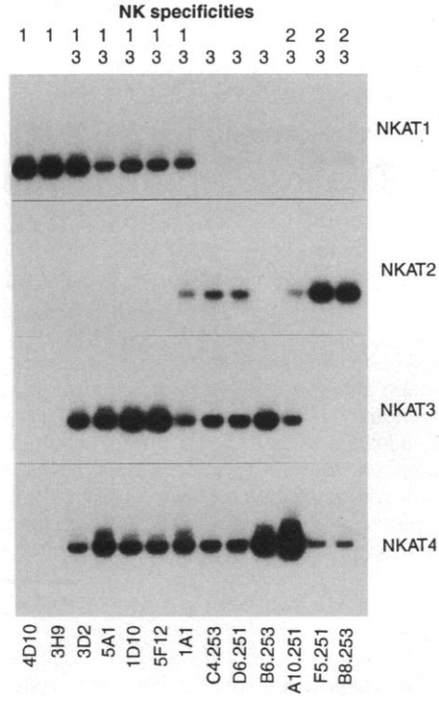

Fig. 4. Expression patterns of NKAT genes in 13 representative NK cell clones (bottom) with different MHC specificities (top); the cell clones were derived from a single donor. NK cell clones were obtained as described (*6*). For determination of MHC specificity, cytotoxicity was tested against HLA-C and HLA-B transfectants in class I deletion mutants by a 4-hour ^{51}Cr-release assay, as described (*6*). Specificities 1, 2, and 3 indicate a significant inhibition of NK cytotoxicity by Asn^{77}-Lys^{80} HLA-C alleles, by Ser^{77}-Asn^{80} HLA-C alleles, or by Ile^{80} HLA-B alleles, respectively. Amplified cDNA obtained from each of the NK clones was tested for NKAT1, 2, 3, and 4 expression by oligotyping (*20*).

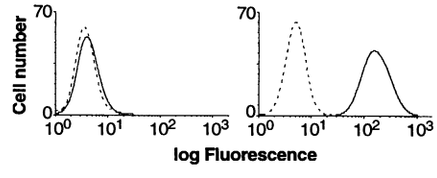

Fig. 5. Cell surface expression of the NKAT1-encoded protein recognized by anti-p58 mAb HP3E4 after transfection of the NKAT1 gene. Jurkat T cells transfected with the pCRIII vector alone (left panel) or with pCRIII-NKAT1 (right panel) were stained with HP3E4 (solid lines) or with control mouse IgM (dashed lines) followed by phycoerythrin-conjugated goat antibody to mouse IgM. Events were collected on a FACScan flow cytometer (Becton-Dickinson).

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10. In our experimental approach, the mRNA differential display technique (*25*) was modified by the use of degenerate primers related to known gene families (family-specific primers) for polymerase chain reaction (PCR). RNAs and oligo(dT)-primed cDNAs were prepared from NK, B, T, and myeloid cells by standard techniques. Ig-SF– and C-type lectin–related primers used for cDNA amplification were designed around consensus sequence positions derived from alignments of Ig- (*26*) and C-type lectin (*27*) amino acid sequences. Primers were 18 to 20 nucleotides (nt) long and included degenerate nucleotides of up to 516-fold degeneracy to ensure efficient priming of unidentified sequences. PCR reactions were done as described (*28*). The cDNA fragments selectively amplified from NK cDNA were gel-purified, cloned into pCRII (Invitrogen), and sequenced by dideoxynucleotide chain termination. The identified sequences were compared with the DNA sequence databases. Selective expression of cDNA clones on NK cells was determined by Northern blot analysis with a panel of RNAs obtained from several tissues and hematopoietic cells. This strategy led to the identification of several genes differentially expressed either in NK cells, in B cells, or in myeloid and dendritic cells.
11. Ig-SF degenerate primers used to amplify this fragment were CCHTGGARCTKGTRRTSACAG-5′ and CCRTAGCAYCYGTAKRTYCC-3′.
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14. Hybridizing transcripts were also detected in the spleen after a long exposure. Polyadenylated [poly(A)$^+$] RNAs from human tissues were purchased from Clontech (Palo Alto, CA). Poly(A)$^+$ RNA from cell lines and cell clones were extracted by standard procedures. RNA samples of 1 μg were fractionated in 1% agarose–2.2 M formaldehyde gel, transferred to nylon membrane by capillary transfer in 10× SSC [1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate (pH 7.2)], and hybridized with ^{32}P-labeled probe. Hybridizations were in 6× SSC, 5 mM EDTA, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.2% SDS, 10% dextran sulfate, and sonicated salmon sperm DNA (100 μg/ml) for 16 hours at 65°C. Membranes were washed two times at room temperature for 30 min each with 3× SSC–0.1% SDS and three times at 65°C for 30 min each with 3× SSC–0.1% SDS, 0.3× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS, respectively, and exposed to Kodak X-Omat AR film at −70°C with intensifying screens.
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18. NKAT1-related transcripts were amplified from cDNA by RT-PCR. cDNA synthesis was as described (*10*). PCR was carried out for 35 cycles, each consisting of 1-min steps at 94°C, 62°C, and 72°C, according to a standard procedure. The primers used for cDNA amplification were CGGCAGCACCATGTCGCTC-5′ (nt 23 to 41) and GTGCTGCGTTAAGAGGGAG-3′ (nt 1441 to 1423). PCR yielded products of ~1.4 kb and ~1.7 kb, which were gel-purified, subcloned into pCRII (Invitrogen), and sequenced by dideoxynucleotide chain termination.
19. To assess the expression and the possible polymorphism of NKAT genes, NKAT transcripts were amplified from NK clones derived from three unrelated donors (*18*) and sequenced. All four NKAT genes were expressed in at least one NK clone for each donor. The sequence of one transcript revealed a variant of NKAT2 (designated NKAT5, accession number L41347), with a charged residue in the transmembrane portion and a shorter cytoplasmic tail without Tyr-x-x-Leu pairs. This variant may have different pairing and signaling properties.
20. For oligonucleotide typing of NKAT genes, an NKAT fragment was amplified by RT-PCR as described (*18*). The primers used for amplification were TTCCCTCCTGGCCCACCCA-5′ (nt 119 to 137 of NKAT1)′ and TCCCTGGATAGATGGTACA-3′ (nt 520 to 502 of NKAT1). Amplified fragments of 402 bp were separated by electrophoresis in a 1.8% agarose gel, transferred to a nylon membrane by capillary blot in 0.4% NaOH, and hybridized with the following ^{32}P-labeled oligonucleotides: TCGCATGACGCAAGACCTGGCAG (NKAT1-specific), CATGATGCAAGACCTTGCAG (NKAT2-specific), CATGATGCTTGCCCTTGCAG (NKAT3-specific), and CCCTTGATGCCTGTCCTTGCA (NKAT4-specific). Hybridization was carried out for 2 hours at 42°C in 5× SSPE [1× SSPE is 0.18 M sodium chloride–0.01 M sodium phosphate (pH 7.4)–0.001 M EDTA], 0.5% SDS, and 5× Denhardt's solution. Membranes were washed in 6× SSC–0.5% SDS for 10 min at room temperature and for 10 min at 65°C, 60°C, 62°C, and 65°C, respectively.
21. NKAT1 was amplified by RT-PCR, cloned into pCRIII (Invitrogen), and transfected by electroporation into Jurkat cell line. Stable transfectants were selected in G418-containing medium and analyzed for cell surface expression of p58 molecules with mAb HP3E4 (*8*). HP3E4-positive cells were sorted on a FACStar plus sorter (Becton Dickinson) and expanded.
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The Role of Igβ in Precursor B Cell Transition and Allelic Exclusion

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Lymphocytes express multicomponent receptor complexes that mediate diverse antigen-dependent and antigen-independent responses. Despite the central role of antigen-independent events in B cell development, little is known about the mechanisms by which they are initiated. The association between the membrane immunoglobulin (Ig) M heavy chain (mμ) and the Igα-Igβ heterodimer is now shown to be essential in inducing both the transition from progenitor to precursor B cells and subsequent allelic exclusion in transgenic mice. The cytoplasmic domain of Igβ is sufficient to induce these early antigen-independent events by a mechanism that requires conserved tyrosine residues in this protein.

Membrane-bound Ig mediates several physiological responses in both developing and mature B lymphocytes (*1*). These responses can be divided into two categories: One set of events is triggered by antigen, whereas a second group is antigen-independent. The antigen-independent responses occur early in the B cell developmental pathway and can be induced by mμ even in the absence of light chain synthesis. The first of these events is a discreet developmental transition from progenitor B cell (pro–B cell) to precursor B cell (pre–B cell). Disruption of either the transmembrane domain–encoding region of the mμ gene or the recombinase activating genes (*RAG*s) that are required for Ig gene assembly results in lymphocytes that fail to develop beyond the pro–B cell stage (*2*, *3*). This developmental deficiency can be specifically complemented by the transgenic addition of a rearranged mμ gene (*4*, *5*). B cells that develop to the pre–B cell stage then undergo heavy-chain allelic exclusion, a second antigen-independent, mμ-mediated response (*6*). Expression of mμ in pre–B cells

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