CD3$^+$CD16$^+$NK1.1$^+$B220$^+$ Large Granular Lymphocytes Arise from both α-βTCR$^+$CD4$^-$CD8$^-$ and γ-δTCR$^+$CD4$^-$CD8$^-$ Cells

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Summary

Cultivation of CD4$^-$CD8$^-$ double negative (DN) mouse thymocytes and splenocytes with recombinant interleukin 2 (IL2) in the absence of other stimulation results in the generation of DN-CD3/TCR$^+$CD16$^+$NK1.1$^+$B220$^+$ large granular lymphocytes (LGL). Purified DNα-βTCR$^+$ thymocytes and splenocytes are CD16$^+$IL2Rα^-IL2Rβ^+NK1.1$^+$B220$^-$CD5^{high}. These cells are unique in that they express both CD16 and T cell receptor (TCR) which are usually mutually exclusive. In addition, they express the natural killer (NK) marker, NK1.1. Cultivation of these cells with IL2 for several days results in the generation of DNα-βTCR$^+$CD16$^+$NK1.1$^+$B220$^+$CD5$^-$ LGL, suggesting that DNα-βTCR$^+$ cells in thymus and spleen are the precursors of the DN LGL reported previously. DNγ-δTCR$^+$CD16$^-$NK1.1$^-$B220$^-$CD5^{high} thymocytes and splenocytes also give rise to DNγ-δTCR$^+$CD16$^+$NK1.1$^+$B220$^+$CD5$^-$ LGL which, as shown previously with DNα-βTCR$^+$ LGL cells, are cytotoxic against NK-sensitive YAC-1 cells. Cytotoxic activity is also induced through either CD16 or the γ-δTCR. DNα-βTCR$^+$ and DNγ-δTCR$^+$ LGL cells are thus similar in phenotype to TCR$^-$ NK cells. DNα-βTCR$^+$ thymocytes express low levels of the γ subunit of the high affinity immunoglobulin E receptor (FcϵRIγ) molecule, an essential component of CD16 expression. FcϵRIγ expression is greatly enhanced after cultivation with IL2, resulting in a higher surface expression of CD16. In contrast to DNα-βTCR$^+$ thymocytes, DNγ-δTCR$^+$ thymocytes do not express detectable CD16 or FcϵRIγ mRNA but expression of both is induced by cultivation with IL2, leading to the expression of CD16 on the surface. Whereas CD16 molecules on both DNα-βTCR$^+$ and DNγ-δTCR$^+$ LGL are associated with only FcϵRIγ homodimers, the TCR on these cells are associated with an FcϵRIγ homodimer and/or CD3ζ-FcϵRIγ heterodimers. These results demonstrate that the FcϵRIγ subunit is a component of the TCR in a fraction of T lineage cells.

T lymphocytes develop from pluripotential hematopoietic stem cells which migrate into the thymus and undergo extensive proliferation and differentiation. Phenotypically, intrathymic precursor cells are characterized by a lack of TCR/CD3, CD4, and CD8 on their cell surface. These CD4$^-$CD8$^-$ double negative (DN)[1] precursor cells lacking TCR/CD3 differentiate to CD4$^+$CD8$^+$ double positive (DP) cells expressing a low level of TCR/CD3 (TCR$^{-/low}$) via a CD3$^-$CD4$^-$CD8$^+$ immature single positive (SP) stage and further differentiate to CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ SP cells expressing a high level of surface TCR/CD3 complex (TCR^{high}) (1). The TCR is responsible for the recognition of Ag/MHC and TCR expression is critical for normal T cell development. The transition from DP to SP cells generating functionally mature Ag/MHC-specific T cells is called thymic selection and is governed by the specificity of the TCR. Cells expressing a TCR specific for class II MHC develop into CD4$^+$ SP cells and those expressing the TCR specific for class I MHC develop into CD8$^+$ SP cells (2–4).

The TCR is a multimolecular complex formed by three groups of transmembrane proteins: (*a*) the clonotype Ag/MHC recognition unit, termed the Tiα-β (or Tiγ-δ) heterodimer (5–9); (*b*) the highly homologous CD3γ, CD3δ, and CD3ϵ subunits (10, 11); and (*c*) the structurally distinct CD3ζ and CD3η subunits which are products of alternative RNA splicing (11, 12). CD3ζ and CD3η form disulfide-linked homo- or heterodimers, thereby creating different TCR isoforms (CD3ζ_2, CD3ζ-η, and CD3η_2) in mouse and are important in targeting partially assembled TCR complexes to the cell surface and transducing stimulatory signals after Ag recognition (11–13).

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; FcϵRIγ, the γ subunit of high affinity IgE receptor; PBS-FG, PBS supplemented with FCS and gentamycin; sIg, surface immunoglobulin; SP, single positive.

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The γ subunit of high affinity IgE receptor (FcϵRIγ) has significant structural homology to CD3ζ and CD3η (14–16). CD3ζ/η and FcϵRIγ are encoded on the same chromosome (mouse chromosome 1), suggesting that CD3ζ/η and FcϵRIγ are derived from a common ancestral gene (17, 18). FcϵRIγ is an essential component of the transmembrane type CD16 expressed on a variety of cells including NK cells (19, 20). Functional similarity between FcϵRIγ and CD3ζ/η has been demonstrated by the ability of members of the CD3ζ/η-FcϵRIγ family to dimerize in distinct receptor systems. CD3ζ is able to complement the formation of a high affinity IgE receptor when mRNAs of FcϵRIα, FcϵRIβ and CD3ζ are microinjected into *Xenopus* oocytes in the absence of FcϵRIγ (21). Human NK cells express CD3ζ as well as FcϵRIγ in association with CD16 in the absence of other TCR components (Tiα-β, CD3$\gamma\delta\epsilon$) (22, 23). These results clearly demonstrate that the CD3ζ/η-FcϵRIγ family functions in the Fc receptor complex. Similarly, transfection of FcϵRIγ into MA5.8, a CD3$\zeta^-\eta^-$ variant of a mouse T cell hybridoma 2B4.11, restored the surface expression of the TCR (24). In addition to these recombinant DNA experiments, recent studies have shown that FcϵRIγ associates with the TCR in some cell types. A long-term mouse CTL line, CTLL, expresses TCRs of at least four different isoforms (25). In addition to conventional CD3ζ/η dimers, heterodimers between FcϵRIγ, CD3ζ, and CD3η are found in CTLL. Long-term IL2-driven mouse splenic LGL cultures, also known as LAK, express a TCR isoform containing a FcϵRIγ homodimer in lieu of CD3ζ/η as part of the TCR molecular complex (26). The same FcϵRIγ containing TCR has been reported in tumor-bearing mice (27). Analysis using a mAb against human FcϵRIγ detected this subunit in association with the TCR on a fraction of peripheral T lymphocytes and thymocytes (28).

Whereas most T lymphocytes undergo thymic selection at the TCR^{low}CD4$^+$CD8$^+$ stage, recent studies have identified a cell population that expresses neither CD4 nor CD8 but expresses high levels of α-βTCR with a skewed V gene repertoire in thymus, spleen, lymph node, peripheral blood, liver, and bone marrow (29–42). Such DNα-βTCR$^+$ lymphocytes contain autoreactive T lymphocytes and are expanded in autoimmune *lpr/lpr* and *gld/gld* mice (29, 43, 44) and in the active stage of human SLE (45). IL2-driven LGL express α-βTCRs but lack CD4 or CD8 (26, 46). These LGL cells are T cells because they express TCR. Of note, however, these cells also express CD16 and NK1.1, both of which are considered markers of NK cells, suggesting that these cells are developmentally related to both T and NK cells. In addition, LGL express a novel type of TCR containing FcϵRIγ as mentioned above (26). To examine the origin of IL2-induced LGL, we herein examined the relationship between IL2-induced LGL and DNα-βTCR$^+$ thymocytes and/or splenocytes in adult animals, based on the observation that these LGL cells do not express CD4 or CD8. We found that DNα-βTCR$^+$ thymocytes and splenocytes express CD16 and NK1.1. Furthermore, these cells constitutively express IL2Rβ and can respond to IL2 without other stimulation. In addition, DNγ-δTCR$^+$ thymocytes and splenocytes also respond to IL2 and become morphologically and functionally LGL expressing CD16 and NK1.1, which are not present before IL2 cultivation. Both DNα-βTCR$^+$ and DNγ-δTCR$^+$ LGL use FcϵRIγ as a component of their TCR. The developmental relationship between DNα-βTCR$^+$ LGL, DNγ-δTCR$^+$ LGL, and TCR$^-$ NK cells and the function of the FcϵRIγ subunit in these cells are discussed.

Materials and Methods

Antibodies

PE-conjugated goat anti-mouse Ig(H+L) and PE-conjugated goat anti-rat IgG were obtained from Fischer Scientific Research (Pittsburgh, PA). PE-conjugated GK1.5 (anti-CD4; 47) was obtained from Becton Dickinson & Co. (San Jose, CA). RED613- and RED670-conjugated streptavidin were obtained from GIBCO BRL (Gaithersburg, MD). FITC-conjugated M1/69 (anti-heat stable antigen [HSA]; 48) and PE-conjugated 56-5 (anti-CD8β; 49) were obtained from Pharmingen (San Diego, CA). Biotin-conjugated RA3-6B2 (anti-B220; 50) and CG16 (anti-CD5) were obtained from Caltag (San Francisco, CA). Hybridomas producing GK1.5, PC61 (anti-IL2Rα; 51), 53-6 (anti-CD8α; 49) and PK136 (anti-NK1.1; 52) were obtained from the American Type Culture Collection (Rockville, MD). Biotin-conjugated TMβ-1 (anti-IL2Rβ; 53) was a kind gift from M. Miyasaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Hybridomas producing RM2-2 (anti-CD2; 54), 2.4G2 (anti-FcγRII/III; 55), 3A10 (anti-Cδ; 56), 145-2C11 (2C11, anti-CD3ϵ; 57), 500A2 (anti-CD3ϵ; 58), H57-597 (H57, anti-Cβ; 59), and F23.1 (anti-Vβ8.1, 2, 3; 60) were kind gifts from H. Yagita (Juntendo University, Tokyo, Japan), J. Unkeless (Mount Sinai Medical Center, New York), S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA), J. Bluestone (University of Chicago, Chicago, IL), J. P. Allison (University of California, Berkeley, CA), R. Kubo (Cytel, La Jolla, CA), and D. Raulet (University of California, Berkeley, CA), respectively. All mAbs were purified from culture supernatant by affinity chromatography using either protein A–conjugated Sepharose CL-4B or protein G–conjugated Sepharose CL-4B (Pharmacia, Uppsala, Sweden). 3A10, 2C11, H57, and 53-6 were FITC-conjugated with FITC "isomer I" according to the manufacturer's recommendation (Molecular Probes, Eugene, OR). 2.4G2, PC61, PK136, 500A2, 3A10, H57-597, F23.1, and 53-6 were biotinylated using a biotin conjugation kit according to the manufacturer's recommendation (Amersham International, Amersham, Bucks, UK). A mAb 1ζ3A1 (anti-mouse CD3ζ; 61) was purified from ascites fluid by protein A–conjugated Sepharose CL-4B. Rabbit antisera #387 (anti-mouse CD3ζ/η; 62) and γ666 (anti-mouse FcϵRIγ; 25) were generous gifts from R. D. Klausner and J.-P. Kinet (National Institutes of Health (NIH), Bethesda, MD), respectively.

Purification of Subpopulations from Thymus and Spleen

Single cell suspension of thymocytes and splenocytes were obtained from 8–10-wk-old C57BL/6 mice. To obtain DN thymocytes and DN-surface immunoglobulin (sIg)$^-$ splenocytes, 1–2 $\times$ 10^9 cells were suspended in 10 ml of PBS supplemented with 2% FCS and 10 μg/ml gentamycin (GIBCO BRL) (PBS-FG) containing 10 μg/ml each unconjugated GK1.5 and 53-6 mAbs and incubated on ice for 45 min. Thymocytes were washed with PBS-FG and suspended in 10 ml of PBS-FG containing 50 mg goat anti-rat

IgG–conjugated magnetic beads (BioMag; Advanced Magnetics, Cambridge, MA) which corresponds to 50 ml of original suspension. Splenocytes were washed with PBS-FG and resuspended in 10 ml of PBS-FG containing 50 mg goat anti-rat IgG–conjugated BioMag magnetic beads and 50 mg goat anti-mouse Ig(H+L)–conjugated BioMag magnetic beads. The cell mixtures were incubated on ice for 15 min with frequent gentle shaking. Cells that bound magnetic beads were removed by magnet (MACS separator; Miltenyi Biotec, Sunnyvale, CA). Unbound cells were recovered and resuspended in 10 ml of PBS-FG containing the same amount of magnetic beads and the magnetic separation repeated. After two cycles of magnetic separations, the yield of DN thymocytes and DNsIg$^-$ splenocytes was usually 1–2 × 10^7 and 5–6 × 10^7, respectively, and the purity of the cells was usually >95% (see Figs. 1 and 3). To purify α-βTCR$^+$, γ-δTCR$^+$, and TCR$^-$ population, purified cells were further incubated with PE-conjugated goat anti-mouse Ig(H+L) and PE-conjugated goat anti-rat IgG on ice for 30 min. After washing, cells were resuspended in 1 ml of PBS-FG containing 2% normal rat serum and 10 μg/ml each of FITC-conjugated H57 and biotinylated 3A10. After 30 min of incubation, cells were washed with PBS-FG and resuspended in 1 ml of PBS-FG containing 2% normal rat serum and 2.5 μg/ml RED670-conjugated streptavidin for 20 min. After washing the cells, DNα-βTCR$^+$, DNγ-δTCR$^+$, and TCR$^-$ populations were sorted on a FACS® Vantage (Becton Dickinson). The machine was calibrated with total thymocytes unstained or stained with either FITC-conjugated M1/69, PE-conjugated GK1.5, or biotin-conjugated 53-6 followed by RED670-conjugated streptavidin. Purified cells were either examined by multicolor flow cytometric analysis or cultured in RPMI 1640 supplemented with 1% penicillin/streptomycin, 10 μg/ml gentamycin, 2 mM L-glutamine, 1% sodium pyruvate, 1% MEM nonessential amino acids, 10 mM Hepes, 10% FCS (all from GIBCO BRL), 50 μM 2-ME (Sigma Chemical Co., St. Louis, MO) and 10 ng/ml recombinant human IL2 (Takeda Chemical Industries, Osaka, Japan; a generous gift from K. A. Smith, Cornell University, Ithaca, NY). CD4$^+$ SP thymocytes were also obtained by cell sorting. Thymocytes were stained with FITC-conjugated anti-CD8α (53-6) and PE-conjugated anti-CD4 (GK1.5), and the CD4$^+$ SP population was sorted by a FACS Vantage.

Flow Cytometric Analysis

To perform multicolor staining analysis of the freshly purified DN thymocytes and DNsIg$^-$ splenocytes, cells were first incubated with a 1:100 dilution each of PE-conjugated goat anti-mouse Ig(H+L) and PE-conjugated goat anti-rat IgG on ice for 30 min. Cultured cells were incubated with a 1:100 dilution of PE-conjugated GK1.5 and PE-conjugated 53-5 on ice for 30 min. After washing, cells were resuspended in 1 ml of PBS-FG containing 2% normal rat serum and 10 μg/ml each of FITC-conjugated H57 for the α-βTCR$^+$ population or FITC-conjugated 3A10 for the γ-δTCR$^+$ population and the following biotinylated mAbs at 10 μg/ml: 2.4G2, TMβ-1, PC61, PK136, 500A2, RA3-6B2, CG16, 3A10 (negative control for the α-βTCR$^+$ population), and H57 (negative control for the γ-δTCR$^+$ population). To block Fc receptor–mediated binding of the mAbs at this staining step, 20 μg/ml unconjugated 2.4G2 was added except for the 2.4G2 staining. After 30 min of incubation, cells were washed with PBS-FG and suspended in 1 ml of PBS-FG containing 2% normal rat serum and 2.5 μg/ml RED-613- or RED670-conjugated streptavidin for 20 min. Flow cytometry was performed with a FACScan® (Becton Dickinson) calibrated as described above. For each sample, 25,000–50,000 events were collected and data analyzed on a computer with a LYSYS II program (Becton Dickinson). An electronic gate for live cells was set through the window of the forward and side scatter profiles. By examining the pattern of FITC staining versus PE staining, DNα-βTCR$^+$ or DNγ-δTCR$^+$ thymocytes and DNsIg$^-$$\alpha$-$\beta$TCR$^+$ or DNsIg$^-$$\gamma$-$\delta$TCR$^+$ splenocytes were then gated and further examined for the RED613 or RED670 staining patterns. In some cases, the staining patterns were shown by two-dimensional plots of FITC versus RED613 or RED670 staining.

Immunoprecipitation and Western Blot Analysis

Cells were lysed at 1–2 × 10^7 cells/ml in TBS (150 mM NaCl, 20 mM Tris/HCl, pH 7.5) containing 1% digitonin, 10 mM iodoacetamide, 5 μg/ml leupeptin, 1 mM PMSF, and 0.24 Trypsin inhibitory unit (TIU)/ml aprotinin (all from Sigma Chemical Co.) by rotating at 4°C for 2 h. Postnuclear supernatant was incubated overnight at 4°C with 20 μl of packed CNBr-activated Sepharose CL-4B beads coupled with various antibodies: 3A10, H57, 2.4G2, and 1ζ3A1 (4–5 mg/ml beads). The bead–antibody–antigen complexes were pelleted by centrifugation, the supernatant removed, and the beads washed once with 15 ml of 0.1% digitonin in TBS, three times with 1 ml of 0.1% digitonin in TBS, once with 1 ml of TBS, and 1 ml of 20 mM Tris/HCl, pH 7.5. Antigen–antibody complexes were solubilized in 20 μl of nonreducing Laemmli's sample buffer at 100°C for 3 min and resolved by two-dimensional nonreducing-reducing SDS-PAGE using 12.5 and 14% acrylamide in the 1st and 2nd dimensions, respectively. After two-dimensional SDS-PAGE, proteins were transferred to nitrocellulose (Bio-Rad Laboratories, Richmond, CA) for 1 h at 100 V in a solution consisting of 25 mM Tris, 192 mM glycine, and 20% MeOH. After a 2-h room temperature incubation in TBS containing 5% FCS and 10 mM NaN_3, blots were incubated for 1 h at room temperature with antibody #387 or γ666 diluted 1:200 in TBS containing 5% FCS and 10 mM NaN_3. Finally, immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit obtained from Amersham International. Prestained molecular weight markers (Bethesda Research Laboratories, Bethesda, MD) were used for the reference: 44 kD, OVA; 29 kD, carbonic anhydrase; 13 kD, lysozyme.

PCR Analysis

Total cellular RNA was prepared from 0.4–1 × 10^6 cells by the vanadyl ribonuclease complex method (63). cDNA copies were produced from total cellular RNA using an oligo dT primer and AMV reverse transcriptase (Molecular Genetics Resources, Tampa, FL) and used as templates for PCR with specific primers as listed below on a Techne thermocycler using the Gene Amp Kit reagents (Perkin Elmer Cetus, Norwalk, CT) for 40 cycles.

FcγRII/III. The sense amplimer 5'GGTGCCATAGCTGGA GGAAC3' located at bp 470-489 of FcγRIII and the antisense amplimer 5'GGAGGCACATCACTAGGGAG3' at bp 733-714 in the transmembrane region of FcγRIII (numbers are according to reference 64) were used to detect FcγRIII (CD16). The PCR product of FcγRIII is a 264-bp fragment. To identify FcγRIIb$_1$ (lymphocyte form) and FcγRIIb$_2$ (monocyte form), the same sense amplimer was used with the antisense amplimer 5'GCAGCTTCT-TCCAGATCAGG3' which lies at bp 1232-1213 of FcγRIIb$_1$, 3' to the 138-bp insertion found in FcγIIb$_1$ as compared with FcγIIb$_2$. Amplification of FcγIIb$_1$ and FcγIIb$_2$ cDNAs produce DNA fragments of 484 and 346 bp, respectively. For PCR, the denaturing, annealing, and extension were performed at 94°C for 1 min, 60°C for 1 min and 72°C for 0.5 min, respectively.

The products were run on a 1.5% agarose gel, alkaline blotted to Zeta-Probe membrane (Bio-Rad Laboratories) and hybridized to the oligonucleotide 5'GCCTGTCACCATCACTGTCC3' at bp 642-661 of FcγRIII and bp 921-940 of FcγRIIb$_1$ and FcγRIIb$_2$. The oligonucleotide was labeled by 5' phosphorylation using polynucleotide kinase and γ-[^{32}P]ATP. Hybridization was performed in 6× SSC, 5× Denhardt's solution, 10 μg/ml denatured salmon sperm DNA, and 0.1% SDS at 52°C. The blot was then washed for 20 min in 6× SSC–0.1% SDS at 52°C and exposed at −70°C to Kodak X-Omat AR x-ray film.

FcεRIγ. The sense amplimer 5'TGATCGCCAGCTCCCAGCGC3' located at bp 1-20 and the antisense amplimer 5'GTGAGAGTCGAGGATCAGGG3' at bp 530-511 in the FcεRIγ cDNA sequence (15) were used. The PCR product of FcεRIγ is a 530-bp fragment. The denaturing, annealing, and extension were performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 0.5 min, respectively. An oligonucleotide 5'GACCTGGATCTTGAGTCGAC3' at bp 192-173 was used for hybridization as above.

CD3ζ. The sense amplimer 5'AGAAGCCTACACTGAGATCG3' located at bp 462-481 and the antisense amplimer 5'GGATGACGTTCTGTGTTCAG3' at bp 774-755 in the CD3ζ cDNA sequence (14) were used. The PCR product of CD3ζ is a 313-bp fragment. The denaturing, annealing, and extension were performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 0.5 min, respectively. An oligonucleotide 5'GTCTCAGCACTGCCACC3' at bp 533-549 was used for hybridization as above.

CD3δ. The sense amplimer 5'GGAACACAGCGGGATTCTGG3' located in the first exon (65) and the antisense amplimer 5'CACCAGCCATGGTGCCCGAG3' in the third exon (bp 289-270 in the CD3δ cDNA sequence; 66) were used. The PCR product of CD3δ is a 319-bp fragment. The denaturing, annealing, and extension were performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 0.5 min, respectively.

Cytotoxic Assay

Target cells were labeled with ^{51}Cr (100 μCi/10^6 cells) for 1 h at 37°C. Targets were then washed three times and added to V-bottom microtiter plates at 5,000 cells/well in RPMI 1640 containing 10% FCS and 10 ng/ml IL2. Effector cells were added at the indicated ratios in a final volume of 180 μl. Plates were centrifuged at 800 rpm for 5 min and then incubated for 4 h at 37°C. After recentrifugation at 2,000 rpm for 2 min, 90 μl were removed from each well for assay of gamma radioactivity. Percent specific lysis was calculated according to the formula $100 \times [(E - C)/(M - C)]$ where E is the experimental value in cpm, C is the control value, and M is the maximum release value. C was determined as the average release in control wells from which effector cells were omitted. M was determined as the average release in wells to which 1% NP-40 was added in place of effector cells. All determinations were performed in triplicate.

Miscellaneous

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred in the animal facility at the Dana-Farber Cancer Institute. Con A blasts were prepared by stimulation of lymph node cells with 5 μg/ml Con A for 24 h followed by cultivation of the stimulated cells in the presence of 10 ng/ml IL2 for 4 d. Light microscopy was performed under a Leitz Labovert microscope equipped with a Hoffmann modulation contrast system.

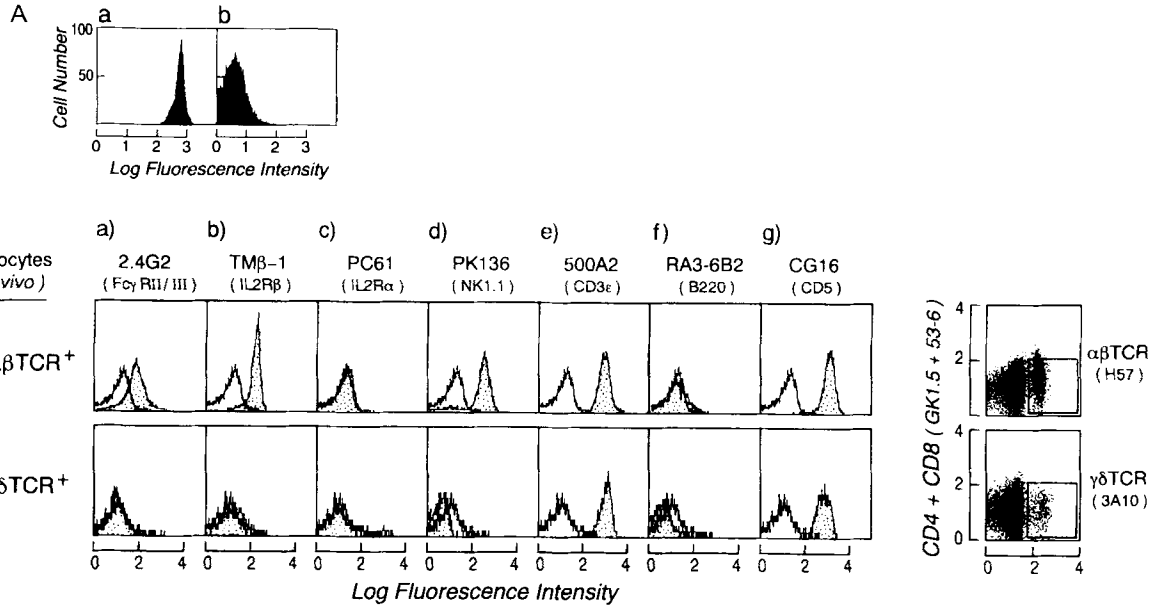


Figure 1. Flow cytometric analysis of DN thymocytes. (*A*) DN thymocytes were prepared from C57BL/6 thymocytes by a magnetic purification method. Cells before (*a*) and after (*b*) purification were examined for their expression of CD4/CD8 by incubation of the cells with mAbs against CD4 (GK1.5) and CD8α (53-6) followed by incubation with PE-conjugated goat anti-rat IgG. Purified DN thymocytes were then stained with FITC-conjugated H57 for α-βTCR$^+$ population (*B*) or FITC-conjugated 3A10 for γ-δTCR$^+$ population (*C*) and the following biotinylated mAbs: (*a*) 2.4G2 (anti-FcγRII/III), (*b*) TMβ-1 (anti-IL2Rβ), (*c*) PC61 (anti-IL2Rα), (*d*) PK136 (anti-NK1.1), (*e*) 500A2 (anti-CD3ε), (*f*) RA3-6B2 (anti-B220), and (*g*) CG16 (anti-CD5). 3A10 (anti-Cδ) and H57 (anti-Cβ) were used as negative controls for the α-βTCR$^+$ and the γ-δTCR$^+$ populations, respectively. To block Fc receptor-mediated binding of the mAbs, unconjugated 2.4G2 was added except for the 2.4G2 staining. At the end, cells were incubated with RED670-conjugated streptavidin as described in Materials and Methods. Flow cytometry was performed with a FACScan® (Becton Dickinson) and data analyzed on a computer with a LYSYS II program. An electronic gate for live cells was set through the window of the forward and side scatter profiles. By examining the pattern of FITC staining versus PE staining, DNα-βTCR$^+$ or DNγ-δTCR$^+$ thymocytes were then gated (*squares*, the *right*) and further examined for the RED670 staining patterns (*left*). Histograms are presented as overlays with negative control stainings.

Results

Purification and Surface Phenotypes of CD4⁻CD8⁻TCR^{high} Thymocytes and Splenocytes. To examine the developmental and functional relationships between DN-CD3⁺CD16⁺ LGL and DN-TCR^{high} thymocytes or peripheral DN T cells, we first purified and characterized DN-TCR^{high} thymocytes. Using repeated depletion of CD4⁺SP, CD8⁺SP, and DP cells from thymocytes by magnetic separation with rat mAbs against CD4/CD8 and magnetic beads conjugated with goat anti-rat IgG, more than 97% of CD4 and/or CD8 expressing cells were removed (Fig. 1 *A*). The surface phenotype of purified DN thymocytes was examined by multicolor flow cytometry. As shown in Fig. 1 *B*, the DNα-βTCR⁺ cells express FcγRII/III defined by mAb 2.4G2 (*a*) and NK1.1 defined by mAb PK136 (*d*), neither of which is expressed on conventional T lymphocytes (data not shown). These cells also express a high level of CD5 defined by mAb CG16 (*g*) and CD2 defined by mAb RM2-2 (data not shown), indicating that these cells share phenotypes characteristic of mature T lymphocytes. Whereas these cells express a high level of IL2Rβ defined by mAb TMβ-1 (Fig. 1 *B*, panel *b*) no IL2Rα was detected by mAb PC61 (Fig. 1 *B*, panel *c*). Staining of these cells with mAb F23.1 reacting with the Vβ8 family showed that nearly 50% of the cells express members of the Vβ8 family as reported (data not shown; 30). In contrast, the DNγ-δTCR⁺ thymocytes (Fig. 1 *C*) express high levels of CD5 and CD2 (data not shown) but FcγRII/III, NK1.1, and IL2Rβ are not detected on these cells. Both populations are B220⁻ as examined by mAb RA3-6B2.

To purify DN-TCR^{high} splenocytes, magnetic beads conjugated with goat anti-mouse Ig(H+L) were used to remove B lymphocytes in addition to rat mAbs against CD4/CD8 and magnetic beads conjugated with goat anti-rat IgG to remove CD4⁺ and CD8⁺ cells. Staining of purified DN cells with H57 (anti-Cβ; anti-α-βTCR) and 500A2 (anti-CD3ϵ) reveals that these cells contain at least three populations: DNα-βTCR⁺, DNγ-δTCR⁺, and TCR⁻ populations (Fig. 2 *A*). The TCR⁻ cells in thymus and spleen likely represent immature thymocytes and NK cells, respectively. DNα-βTCR⁺ splenocytes have a nearly identical surface phenotype to that of DNα-βTCR⁺ thymocytes except that 30–40% of the DNα-βTCR⁺ splenocytes do not express NK1.1 (Fig. 2 and data not shown).

Induction of LGL from DN Thymocytes and Splenocytes by IL-2. Because the β subunit of the IL2R is thought to transmit proliferative signals, the DNα-βTCR⁺ cells may be the precursors of the IL-2-induced LGL observed previously (26, 46). To examine this, purified DN thymocytes and splenocytes were cultured in the presence of rIL-2. After sev-

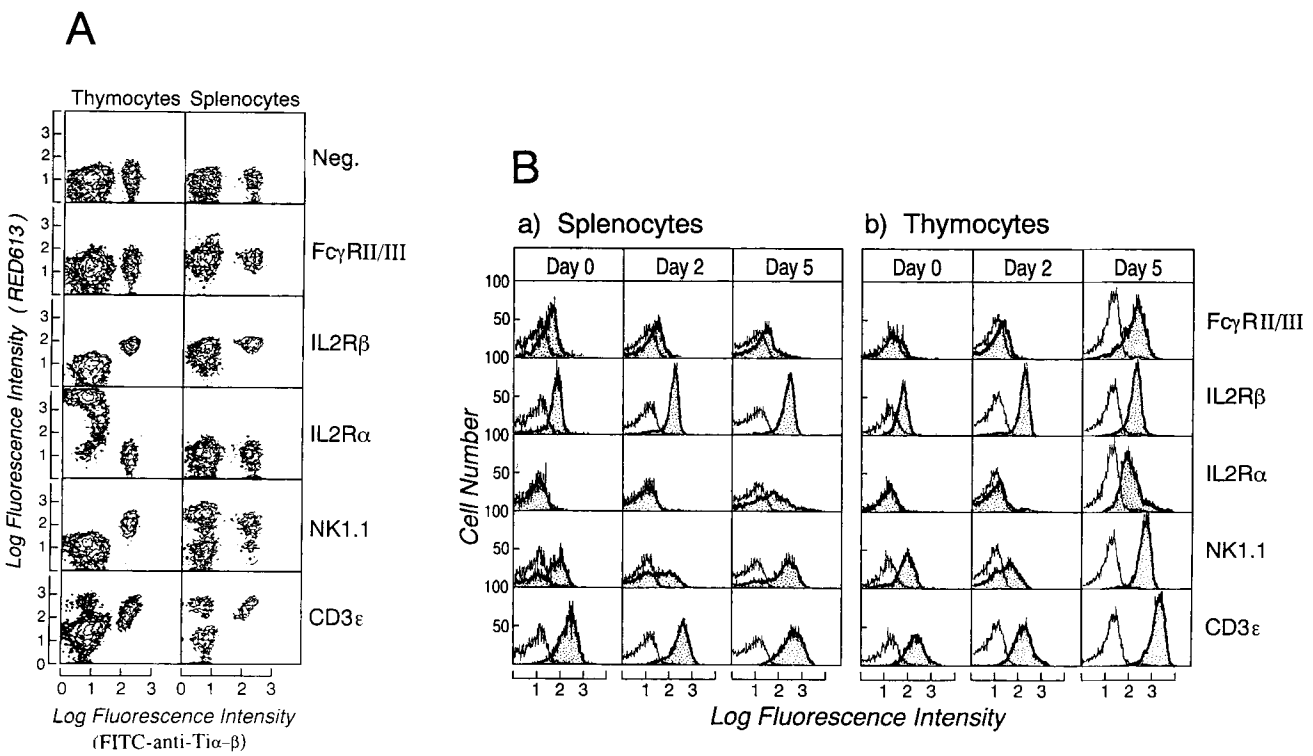


Figure 2. Induction of LGL by IL2 from purified DN thymocytes and DNsIg⁻ splenocytes. (*A*) DNsIg⁻ thymocytes and splenocytes were purified by magnetic separation and then stained with FITC-conjugated H57 and the following biotinylated mAbs: 2.4G2 (anti-FcγRII/III), TMβ-1 (anti-IL-2Rβ), PC61 (anti-IL2Rα), PK136 (anti-NK1.1), and 500A2 (anti-CD3ϵ). An irrelevant mAb was used as a negative control. The final staining was with RED613-conjugated streptavidin. Two-color profiles for FITC and RED613 of fresh DNsIg⁻ cells are shown. (*B*) Purified DNsIg⁻ splenocytes (*a*) and thymocytes (*b*) were cultured in the presence of IL2 for the indicated time periods. Cells were first stained with PE-conjugated mAbs against CD4 (GK1.5), CD8β (53-5), and PE-conjugated goat anti-mouse Ig(H+L) and then stained with FITC-conjugated H57 and various biotinylated mAbs as indicated (*A*). The DNα-βTCR⁺ fraction was gated and staining patterns of RED613 on indicated days during IL2 cultivation are shown.

eral days of cultivation with IL2, cells morphologically characteristic of LGL were found to be proliferating. Multicolor flow cytometric analysis revealed that the DNα-βTCR$^+$FcγRII/III$^+$NK1.1$^+$ cells from both thymus and spleen were induced to express IL2Rα by day 5 and were indistinguishable from the long-term IL2-driven LGL reported previously (Fig. 2 *B*; and 26).

It is possible that these LGL cells result from proliferation of a fraction of TCR$^-$ immature cells that express IL2R, respond to IL2, and then express α-βTCR. To rule out this possibility and to directly examine whether DNα-βTCR$^+$ thymocytes are the precursors of DNα-βTCR$^+$ LGLs, we further purified these populations by cell sorting. As shown in Fig. 3 *A*, DN thymocytes purified by magnetic separation were further stained with FITC-conjugated H57 (anti-α-βTCR) and biotin-conjugated 3A10 (anti-γ-δTCR) followed by RED670-conjugated streptavidin and DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes were separately sorted. Reanalysis after sorting showed that both populations were >95% pure (Fig. 3 *A*). When these purified cells were cultured in the presence of IL2, most of the cells survived and started to proliferate within a few days. After 2 wk of cultivation, cells determined by morphology to be LGL were induced from DNα-βTCR$^+$ thymocytes (Fig. 4). To our surprise, DNγ-δTCR$^+$ cells also responded to IL2 and gave rise to LGL of similar morphology (Fig. 4). These results suggest that both DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes respond to IL2 and differentiate to LGL. Furthermore, both cells developed a similar phenotype, namely FcγRII/III$^+$NK1.1$^+$ (Fig. 3, *B* and *C*). As shown below, the FcγR on these cells is CD16. In contrast to the cells before cultivation, these cells express a high level of B220 and lose the expression of CD5. These results indicate that DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes can proliferate in response to IL2 in the absence of TCR stimulation and become LGLs. We therefore designate these cells as thymic α-βTCR$^+$ and γ-δTCR$^+$ LGL.

Since the surface phenotypes of DNα-βTCR$^+$ and DNγ-δTCR$^+$ splenocytes were nearly identical to those of DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes, respectively, except that DNγ-δTCR$^+$ splenocytes express low levels of IL2Rβ (Fig. 2 and data not shown), we next examined the response of DNα-βTCR$^+$ and DNγ-δTCR$^+$ splenocytes to IL2. α-βTCR$^+$, γ-δTCR$^+$, and TCR$^-$ cells were further purified from CD4$^-$CD8$^-$ sIg$^-$ cells by cell sorting as described above and cultured with IL2. Again, cells of LGL morphology were induced within a week of cultivation. Flow cytometric analysis showed that these cells were indistinguishable from those obtained from thymocytes (Fig. 5). Both α-βTCR$^+$ (Fig. 5 *A*) and γ-δTCR$^+$ LGL (Fig. 5 *B*) were FcγRII/III$^+$ NK1.1$^+$B220$^+$CD5$^-$. These phenotypes were identical to those of NK cells (Fig. 5 *C*) except for the expression of the

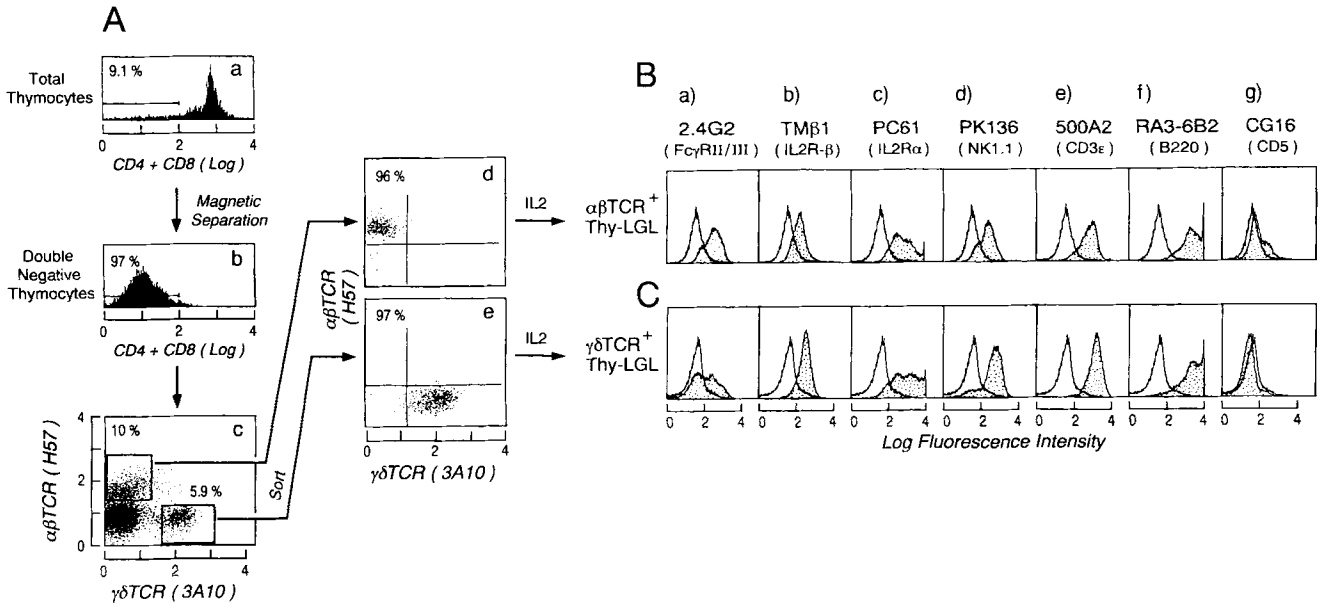


Figure 3. Induction of LGL from purified DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes by IL2. (*A*) Purification of DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes. Thymocytes (8×10^8) obtained from nine C57BL/6 mice were incubated with mAbs against CD4 (GK1.5) and CD8α (53-6) and 10^7 DN thymocytes were recovered by magnetic separation. The purity was 97% as evident by the comparison of the staining patterns between before (*a*) and after (*b*) separation with mAbs against CD4 (GK1.5) and CD8α (53-6) followed by PE-conjugated goat anti-rat IgG. Purified DN thymocytes were further stained with FITC-conjugated H57 (anti-Cβ) and biotinylated 3A10 (anti-Cδ) followed by RED670-conjugated streptavidin (*c*). DNα-β-TCR$^+$ and DNγ-δTCR$^+$ thymocytes were then sorted by a FACS® Vantage and 8.5×10^5 and 2.9×10^5 cells were recovered for DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes, respectively. The purity of DNα-βTCR$^+$ (*d*) and DNγ-δTCR$^+$ (*e*) thymocytes was 96 and 97%, respectively. (*B* and *C*) Purified DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes were cultured in the presence of IL2 for 9 d. Resulting α-βTCR$^+$ (*B*) and γ-δTCR$^+$ (*C*) LGL cells were then stained with various biotinylated antibodies as indicated, followed by RED670-conjugated streptavidin as described in Fig. 2. (*a*) 2.4G2 (anti-FcγRII/III), (*b*) TMβ-1 (anti-IL2Rβ), (*c*) PC61 (anti-IL2Rα), (*d*) PK136 (anti-NK1.1), (*e*) 500A2 (anti-CD3ϵ), (*f*) RA3-6B2 (anti-B220), and (*g*) CG16 (anti-CD5), 3A10 (anti-Cδ; negative control for the α-βTCR$^+$ LGL), H57 (anti-Cβ; negative control for the γ-δTCR$^+$ LGL).

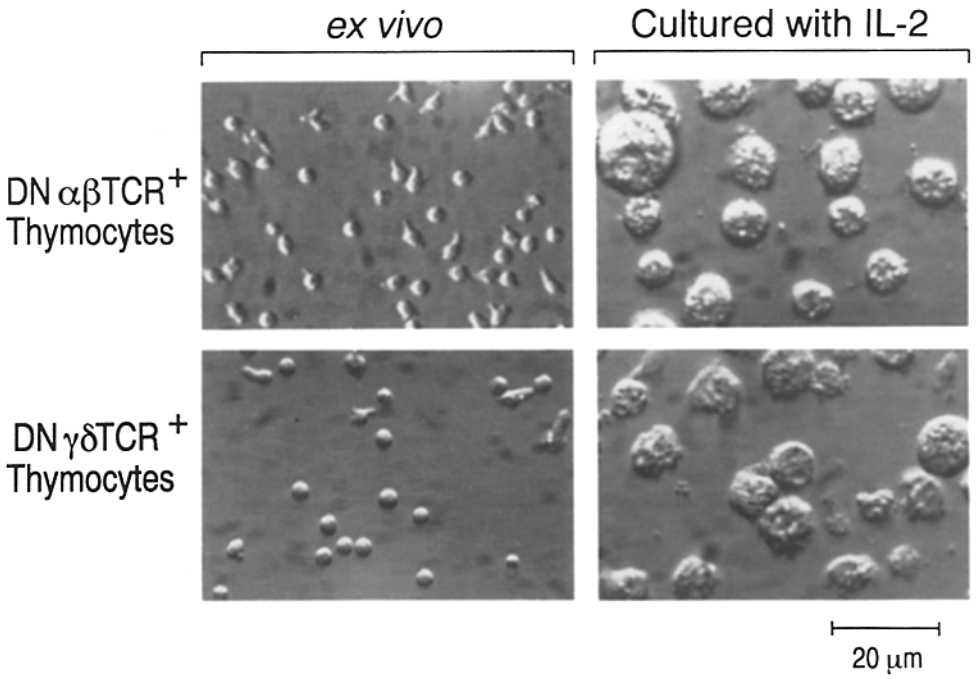


Figure 4. Morphology of LGL cells.DNα-βTCR$^+$ thymocytes, DNγ-δTCR$^+$ thymocytes, α-β TCR$^+$, and γ-δTCR$^+$ LGL cells obtained from the purified DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes with IL2 were examined under a Leitz Labovert microscope equipped with a Hoffmann modulation contrast system and a 20× objective lens. Bar, 20 μm.

TCR. We designate these cells as splenic α-βTCR$^+$ and γ-δTCR$^+$ LGL. From the above results, we conclude that IL2 stimulation of DNα-βTCR$^+$ and DNγ-δTCR$^+$ cells from both thymus and spleen generates LGL cells. Whereas DNα-βTCR$^+$ splenocytes have 30–40% of cells that do not express NK1.1, DNα-βTCR$^+$ LGL are nearly 100% NK1.1$^+$. It is unknown whether only NK1.1$^+$ cells expand or NK1.1$^-$ cells acquire NK1.1 expression in response to IL2.

Cytotoxic Activity of γ-δTCR$^+$ LGL. NK cells show strong cytotoxic activity against certain tumor cells such as YAC-1 cells and, in addition, display antibody-dependent cellular cytotoxicity (ADCC) through CD16. The α-βTCR$^+$ LGL show similar cytotoxic activity against NK-sensitive YAC-1 cells. Furthermore, cytotoxic activity is induced through both CD16 and the TCR (26). Since γ-δTCR$^+$ LGL cells are phenotypically similar to both NK and α-βTCR$^+$ LGL cells, we next examined the cytotoxic activity of these cells. As shown in Fig. 6, splenic γ-δTCR$^+$ LGL cells show strong cytotoxic activity against YAC-1 cells.

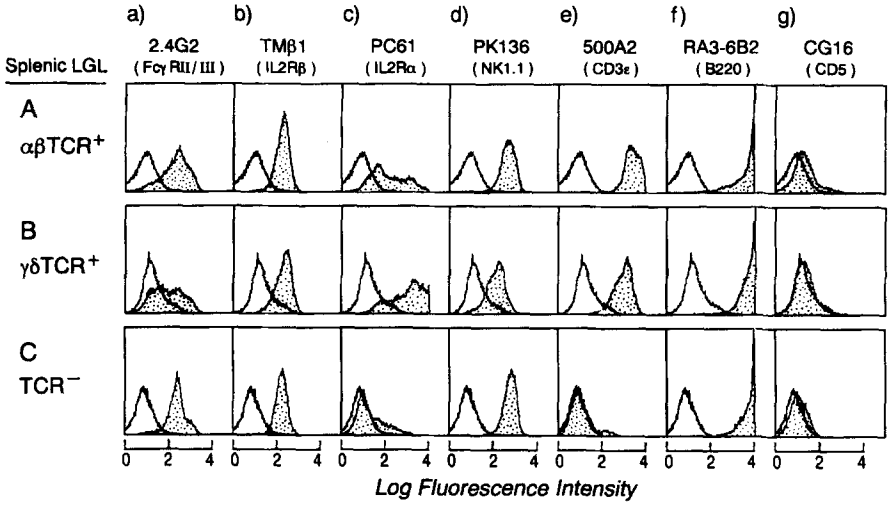


Figure 5. Phenotypes of LGL cells obtained from purified DNα-βTCR$^+$, DNγ-δTCR$^+$ and DN-TCR$^-$ splenocytes. DNsIg$^-$ splenocytes were purified by magnetic separation and further stained with FITC-conjugated H57 (anti-Cβ) and biotinylated 3A10 (anti-Cδ) followed by RED670-conjugated streptavidin as shown in Fig. 3 *A*. DNα-βTCR$^+$ (*A*), DNγ-δTCR$^+$ (*B*), and DN-TCR$^-$ (*C*) cells were then sorted by a FACS®Vantage and 5 × 10^5 cells with a purity of >85% were obtained for each population. Purified cells were then cultured in the presence of IL2. On day 12, cells were stained with various biotinylated antibodies as indicated followed by RED670-streptavidin as described in Fig. 2. (*a*) 2.4G2 (anti-FcγRII/III), (*b*) TMβ-1 (anti-IL2Rβ), (*c*) PC61 (anti-IL2Rα), (*d*) PK136 (anti-NK1.1), (*e*) 500A2 (anti-CD3ε), (*f*) RA3-6B2 (anti-B220), and (*g*) CG16 (anti-CD5), 3A10 (anti-Cδ; negative control for the α-βTCR$^+$ LGL and TCR$^-$ LGL), H57 (anti-Cβ; negative control for the γ-δTCR$^+$ LGL). TCR$^-$ LGL cells were also stained with FITC-conjugated 2C11 to exclude a fraction (~10%) of cells expressing CD3/TCR.

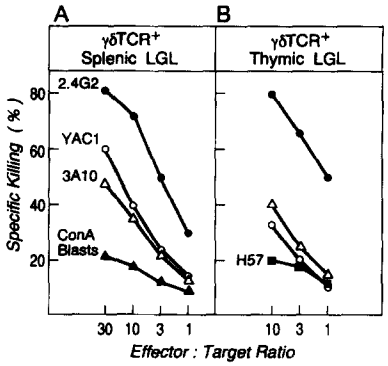


Figure 6. Cytotoxic activity of γ-δTCR+ LGL. Splenic γ-δTCR+ LGL (*A*) and thymic γ-δTCR+ LGL (*B*) were obtained from purified DNγ-δTCR+ splenocytes and thymocytes by cultivation with IL2 for 2 and 3 wk, respectively. Cytotoxic activities against YAC-1 (○), 2.4G2 hybridoma (●), 3A10 hybridoma (△), autologous (C57BL/6) Con A blasts (▲, *A*), or H57 hybridoma (■, *B*) were examined by a standard ^{51}Cr-release assay with the indicated effector/target ratios.

Cytotoxic activities were also induced through FcγR and TCR molecules examined by redirected cytolysis against B cell hybridomas producing mAbs 2.4G2 (anti-FcγRII/III) and 3A10 (anti-Cδ), respectively. Thymic γ-δTCR+ LGL cells also showed cytotoxic activities, although the activities were weaker than those of splenic LGL cells. These results indicate that the γ-δTCR+ LGL cells, like α-βTCR+ LGL cells, have cytotoxic activity similar to that of NK cells.

PCR Analysis of FcγR Isotype and Expression of FcεRIγ and CD3ζ. The mAb 2.4G2 is known to recognize three distinct FcγR isotypes, FcγRIIb1 (lymphocyte form), FcγRIIb2 (monocyte form), and FcγRIII (CD16) (64, 67, 68). We thus examined the expression of these molecules in DN thymocytes and LGL cells by reverse PCR analysis as described previously (26). To this end, cDNAs were synthesized from total RNA prepared from 0.5–1 × 10^6 cells and PCR was performed with amplimers specific for each molecule. As shown in Fig. 7, FcγR on DNα-βTCR+ thymocytes, α-βTCR+ LGL, and γ-δTCR+ LGL is exclusively of the CD16 form whereas unfractionated DN thymocytes express all three FcγR isotypes (Fig. 7, panels d and e). In contrast to DNα-βTCR+ thymocytes or LGLs, DNγ-δTCR+ thymocytes do not express any FcγR isotypes as expected from the flow cytometric analysis (Fig. 1 *C*). PCR amplimers specific for FcεRIγ detected the expression of FcεRIγ mRNA in unfractionated DN thymocytes, DNα-βTCR+ thymocytes, α-βTCR+ LGL and γ-δTCR+ LGL (Fig. 7 *c*). The amount of FcεRIγ mRNA is, however, much higher (>20-fold) in

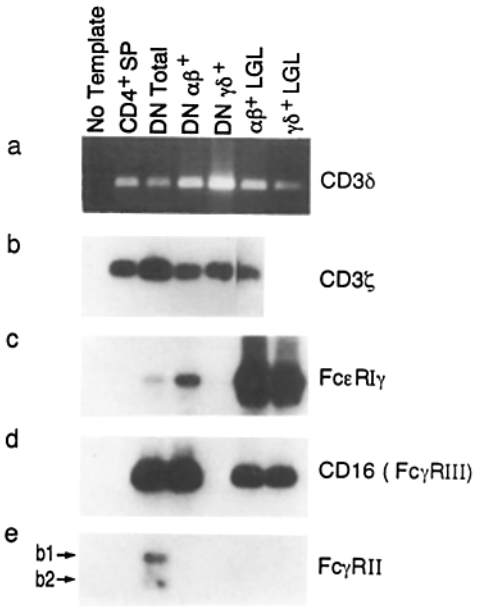
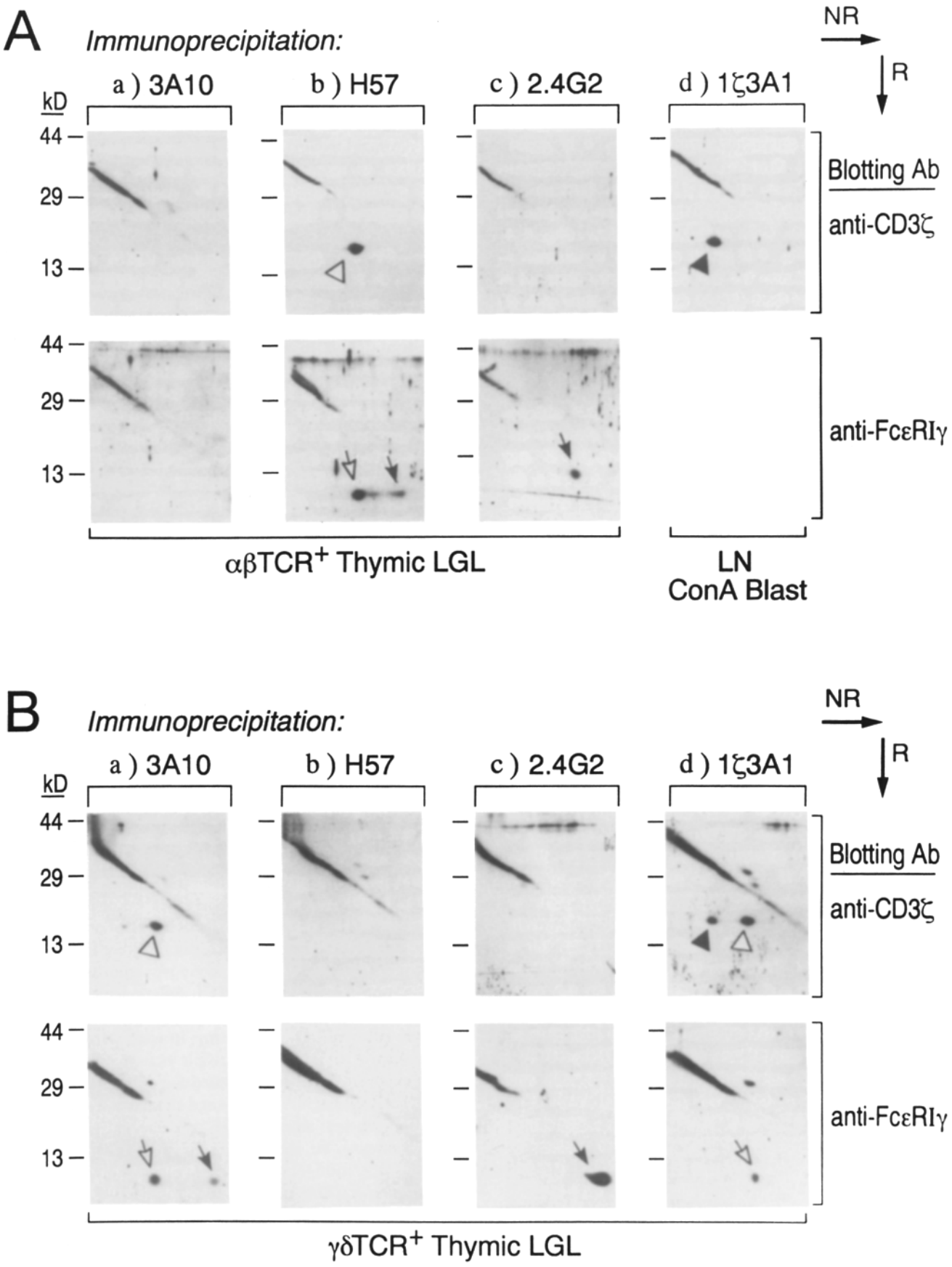


Figure 7. PCR analysis of FcγR isotype and expression of FcεRIγ and CD3ζ. Total cellular RNA was prepared from 0.4–1 × 10^6 CD4+ SP thymocytes, unfractionated DN thymocytes, DNα-βTCR+ thymocytes, DNγ-δTCR+ thymocytes, α-βTCR+ LGL, and γ-δTCR+ LGL cells. cDNA copies were produced from total cellular RNA using an oligo dT primer and AMV reverse transcriptase and used as a template for PCR with primers for CD3δ (*a*), CD3ζ (*b*), FcεRIγ (*c*), CD16/FcγRIII (*d*), or FcγRII (*e*) as described in Materials and Methods. PCR products from 10^4 cells were resolved on a 1.5% agarose gels and visualized by ethidium bromide staining (*a*) or Southern blotting followed by hybridization to internal oligonucleotides (*b–e*) as described in Materials and Methods. Films were exposed for 2 h except that those for (*e*) and α-βTCR+ LGL (*b*) were exposed for 12 h. (*b1* and *b2*, *e*) Migration positions for PCR products from $FcγRIIb_1$ (lymphocyte form) and $FcγRIIb_2$ (monocyte form), respectively.

LGLs than in DNα-βTCR+ thymocytes. A longer exposure of the film showed a small amount of FcεRIγ mRNA expression in DNγ-δTCR+ thymocytes but no detectable band was obtained from CD4+SP thymocytes (data not shown). Finally, CD3ζ and CD3δ mRNAs were observed in all cells tested including CD4+ SP thymocytes (Fig. 7, *a* and *b*). These results indicate that both CD16 and FcεRIγ molecules are expressed in DNα-βTCR+ thymocytes and their expression is induced in DNγ-δTCR+ thymocytes upon IL2 stimulation.

Association of FcεRIγ with TCR and CD16 Molecules on LGL. Since CD3ζ and FcεRIγ are important in the surface expression of both TCR and CD16, we next examined the

Figure 8. Association of FcεRIγ with TCR and CD16 molecules on LGL. Thymus-derived α-βTCR+ LGL (*A*, panels *a–c*) and γ-δTCR+ LGL cells (*B*) and lypmh node–derived Con A blast cells (*A*, panel *d*) were lysed in 1% digitonin lysis buffer solution. Postnuclear supernatants were immunoprecipitated by CNBr-activated Sepharose CL4B beads conjugated with (*a*) 3A10 (anti-Cδ), (*b*) H57 (anti-Cβ), (*c*) 2.4G2 (anti-FcγRII/III), or (*d*) 1ζ3A1 (anti-CD3ζ). Immunoprecipitates were resolved by two-dimensional nonreducing/reducing diagonal gels and Western blotted with rabbit antisera #387 (anti-CD3ζ) or γ666 (anti-FcεRIγ). Proteins were visualized by the enhanced chemiluminescence detection method (Amersham International). Numbers on the left side indicate the migration positions of prestained molecular weight markers (Bethesda Research Laboratories). 44 kD, OVA; 29kD, carbonic anhydrase; and 13 kD lysozyme. (*Open and closed triangles*) CD3ζ in CD3ζ-FcεRIγ heterodimers, and CD3ζ homodimers, respectively. (*Open and closed arrows*) FcεRIγ in CD3ζ-FcεRIγ heterodimers and FcεRIγ homodimers, respectively.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Immunoprecipitation:
NR
R
a) 3A10
b) H57
c) 2.4G2
d) 1ζ3A1
kD
44
29
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Blotting Ab
anti-CD3ζ
anti-FcεRIγ
αβTCR+ Thymic LGL
LN
ConA Blast
B
Immunoprecipitation:
NR
R
a) 3A10
b) H57
c) 2.4G2
d) 1ζ3A1
kD
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29
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Blotting Ab
anti-CD3ζ
anti-FcεRIγ
γδTCR+ Thymic LGL

association of these molecules with TCR and CD16 molecules on α-βTCR$^+$ LGL and γ-δTCR$^+$ LGL. As shown in Fig. 8, CD3ζ-FcϵRIγ heterodimers and FcϵRIγ homodimers were readily detected in association with TCRs on both α-βTCR$^+$ LGL (Fig. 8 *A*, panel *b*) and γ-δTCR$^+$ LGL (Fig. 8, panel *a*), whereas only FcϵRIγ homodimers are observed in the CD16 immunoprecipitates (Fig. 8 *A*, panel *c* and Fig. 8 *B*, panel *c*). Anti-CD3ζ mAb immunoprecipitates both CD3ζ homodimers and CD3ζ-FcϵRIγ heterodimers (Fig. 8 *B*, panel *d*) but little CD3ζ homodimers are detected in association with the TCR or CD16 complexes (Fig. 8 *A*, panels *b* and *c* and Fig. 8 *B* panels *a* and *c*). In contrast to LGL, CD3ζ homodimers but not FcϵRIγ were detected in Con A blasts derived from lymph node cells; these cells do not express FcγR or NK1.1 (Fig. 8 *A*, panel *d* and data not shown).

Discussion

We have previously reported that long-term LGL cultures obtained from splenocytes incubated with IL2 show an unusual surface phenotype coexpressing CD3/α-βTCR and CD16 in the absence of CD4 and CD8 (26). CD16 is expressed on NK cells but not on "conventional" T lymphocytes in adult animals with rare exceptions such as the T cells found in LGL lymphocytosis patients (69, 70). In addition, these cells express NK1.1 and exhibit strong cytotoxic activity against NK-sensitive YAC-1 cells. Cytotoxic activities can be induced through both TCR and CD16 structures. Thus, these LGL cells have characteristics of both T and NK cells. In fetal thymic development, TCR$^-$ fetal thymocytes are nearly 100% CD16$^+$ and differentiate to T lymphocytes within the thymus but differentiate to NK cells when cultured with IL2 in vitro, indicating that CD16$^+$ fetal thymocytes contain precursors of both T cells and NK cells (71). We herein demonstrate that both thymic and splenic DN cells in adult animals contain a population coexpressing α-βTCR, CD16, and NK1.1. These cells become LGL upon IL2 cultivation and exhibit surface phenotypes identical to those of LGL reported previously (26). It is therefore likely that these DNα-βTCR$^+$ cells are the precursors of IL2-induced LGL. In contrast to conventional SP thymocytes or mature T lymphocytes, DNα-βTCR$^+$ cells constitutively express the β subunit of IL2R without expressing the α subunit (Figs. 1 and 2; 72). Since the β subunit of IL2R in combination with the γ subunit is responsible for transmitting signals (73), these cells likely express the γ subunit of IL2R and can respond to IL2 without antigenic stimulation. To our surprise, IL2 stimulation of purified DNγ-δTCR$^+$ cells also generated cells morphologically, phenotypically, and functionally identical to LGL. The purified DNγ-δTCR$^+$ cells do not express CD16 or NK1.1 but acquire expression of these molecules after cultivation with IL2. In contrast to the human γ-δTCR$^+$ cells (74) or DNγ-δTCR$^+$ splenocytes, mouse DNγ-δTCR$^+$ thymocytes express little IL2Rβ as examined by flow cytometry (Fig. 1 *C*) and yet this population respond to IL2 without stimulation through the TCR. It is possible that binding of mAb to the γ-δTCR during cell sorting activates the DNγ-δTCR$^+$ cells and induces the IL2R. This possibility is, however, unlikely because expansion of DNγ-δTCR$^+$ LGL was also observed from unfractionated DN thymocytes (data not shown). It is unknown whether these cells express the IL2Rβ/γ complex at a low level or express a distinct type of IL2R. Although the majority of the purified DNγ-δTCR$^+$ thymocytes survive during IL2 cultivation, we cannot formally rule out the possibility that a small portion of the DNγ-δTCR$^+$ population expressing IL2Rβ respond to IL2, thus producing the DNγ-δTCR$^+$ LGL.

DNα-βTCR$^+$ thymocytes express CD16 mRNA as shown by PCR analysis (Fig. 7). The level of CD16 mRNA expression seems unchanged but the surface expression of CD16 detected by the 2.4G2 mAb is greatly enhanced after IL2 cultivation. CD16 requires FcϵRIγ or CD3ζ for its transportation to the cell surface. In the human, the CD16 molecule can associate with homo- or heterodimers between FcϵRIγ and CD3ζ (22, 23). In contrast, mouse CD16 is unable to associate with CD3ζ either as a homo- or a heterodimer with FcϵRIγ (Fig. 8). This result confirms the previous report that mouse CD3ζ cannot associate with CD16 in transfection experiments (20). The DNα-βTCR$^+$ thymocytes express FcϵRIγ mRNA at a low level whereas CD3ζ mRNA is expressed at a level similar to that of CD4$^+$ SP thymocytes (Fig. 7). The level of the FcϵRIγ mRNA expression is, however, dramatically increased upon IL2 cultivation. It is therefore likely that the IgG binding subunit (CD16) is expressed at the mRNA level but that the level of FcϵRIγ subunit limits CD16 surface expression in this population. In contrast to the DNα-βTCR$^+$ thymocytes, DNγ-δTCR$^+$ thymocytes do not express any detectable level of CD16 mRNA. However, this population is induced to express both CD16 and FcϵRIγ mRNAs after cultivation with IL2 and acquires the surface expression of CD16. It has been reported that CD16 surface expression is induced on Vγ3$^+$ skin intraepithelial lymphocytes (sIEL) after stimulation with Con A and IL2 (75). It is possible from our results that IL2 is the major inducer of CD16 on sIEL.

FcϵRIγ plays an important role in the expression of CD16 as well as FcϵRI and as shown herein, this molecule is also a subunit of TCRs in LGL. The TCRs on LGL cells are associated with CD3ζ-FcϵRIγ heterodimers as well as FcϵRIγ homodimers (Fig. 8). We have previously reported that the TCR on long-term IL2-driven LGL cells is associated with FcϵRIγ homodimers without CD3ζ/η (26). The latter LGL cells were cultured for a longer time in vitro with IL2 than those reported here and it is possible that they lost CD3ζ protein expression after long in vitro cultivation. In fact, the level of FcϵRIγ mRNA is increased dramatically after IL2 cultivation whereas that of CD3ζ is decreased (Fig. 7 and data not shown). At the protein level, LGLs derived from DNα-βTCR$^+$ and DNγ-δTCR$^+$ thymocytes gradually lost the expression of CD3ζ and the amount of the TCR-associated FcϵRIγ homodimer increased (data not shown). It seems therefore likely that the FcϵRIγ homodimer becomes the major component of the TCR after prolonged cultivation of these

cells in IL2. Similar changes in subunit composition have been reported in tumor-bearing mice. Mizoguchi et al. (27) observed that most T cells express TCRs associated with FcεRIγ homodimers in mice after growth of implanted tumors. It is unknown, however, whether these two cases are related.

Although not associated with the TCR, CD3ζ homodimers are also present in the cell as shown by biochemical analysis (Fig. 8). Orloff et al. (25) have reported that the CTLL cell line expresses CD3ζ, CD3η, and FcεRIγ, but the major component of the TCR is the CD3ζ-FcεRIγ heterodimer. It seems likely from these results that the CD3ζ-FcεRIγ heterodimer has a higher affinity for the TCR than the other dimers. In contrast to the TCR, only FcεRIγ homodimers are capable of association with CD16 in the same cell (Fig. 8). We were unable to perform biochemical analysis of the TCR component in freshly isolated DNα-βTCR$^+$ or DNγ-δTCR$^+$ thymocytes because of insufficient cell numbers. However, since the DNα-βTCR$^+$ thymocytes express both FcεRIγ and CD3ζ, it is likely that the TCR on this population contains both FcεRIγ and CD3ζ.

Using a mAb against FcεRIγ, Vivier et al. (28) reported that this subunit is expressed in human thymocytes and peripheral T cells in both CD4 and CD8 subsets and is associated with the TCR. In contrast to the human, as shown here in mouse, mRNA for FcεRIγ was not detected in CD4$^+$ SP cells (Fig. 7) or DP cells (data not shown) by PCR. Malissen et al. (76) and Liu et al. (77) have recently demonstrated that intestinal intraepithelial lymphocytes (iIEL) express TCRs containing FcεRIγ by employing CD3ζ$^-$CD3η$^-$ mice which lack most T lymphocytes due to the deficiency in CD3ζ/η expression. These iIEL cells are known to develop extrathymically and have a distinct selection pathway from that of conventional T lymphocytes (78–80). These results together with our results indicate that the FcεRIγ subunit is expressed in distinct subsets of T cells.

It has been suggested that T and NK cells are of the same developmental origin. Fetal thymocytes can differentiate in vivo to T lymphocytes when transferred into the thymus but differentiate to NK cells when cultured with IL2 in vitro, indicating that fetal thymocytes contain precursors of both T and NK cells (71). It has also been shown that CD16$^+$ NK1.1$^+$TCR$^-$NK cells are induced by cultivation of CD16$^-$NK1.1$^-$ fetal liver cells with IL2 (81), indicating that both CD16 and NK1.1 can be induced by IL2. Our results that IL2 cultivation of DNα-βTCR$^+$ or DNγ-δTCR$^+$ cells from both thymus and spleen results in the generation of LGL that are characteristic of NK cells support the hypothesis that T and NK cells are of same developmental origin. Nearly 100% of day 14.5 fetal thymocytes express CD16 without the CD3/TCR complex but los CD16 expression and acquire CD3/TCR expression upon further development (71). FcεRIγ is associated with CD16 in such early thymocytes but its expression is downregulated during the induction of TCR expression in association with CD3ζ. It is possible that a small population of thymocytes continues to express both CD16 and the FcεRIγ subunit and that such a population is the precursor of the DNα-βTCR$^+$ thymocytes (Fig. 9).

DNα-βTCR$^+$ cells have been observed in both humans and mice. In mice, these cells are not detectably observed during fetal development but appear in the thymus after birth, implying a distinct developmental program for this population (1). DNα-βTCR$^+$ cells have also been observed in various other organs such as spleen, lymph node, bone marrow, peripheral blood, liver, and intestine (29–42). It is unclear, however, whether these cells are of the same origin or are derived from separate developmental pathways in different organs. Thymic dependence is also controversial. For example, DNα-βTCR$^+$ cells are found in the spleen of athymic nude mice but not in bone marrow (35, 38), suggesting that a portion of DNα-βTCR$^+$ cells develop extrathymically but that those in bone marrow require thymus for their development. DNα-βTCR$^+$ cells were observed in the lymph nodes after intrathymic cell transfer of CD3$^-$CD4$^-$CD8$^-$ thymocytes, indicating that at least a portion of DNα-βTCR$^+$ cells are of thymic origin (32). Levitsky et al. (36) showed that thymus engraftment into athymic mice resulted in the development of donor type DNα-βTCR$^+$NK1.1$^+$ cells in periphery and that the DNα-βTCR$^+$NK1.1$^+$ cells preferentially localize to bone marrow. As shown in Fig. 1, only 60–70% of DNα-βTCR$^+$ splenocytes express NK1.1 whereas nearly 100% of DNα-βTCR$^+$ thymocytes express NK1.1, thereby revealing at least two distinct subsets of DNα-βTCR$^+$ cells in spleen. It is therefore possible that there are several different subsets of DNα-βTCR$^+$ cells whose development is either dependent or independent of thymus (Fig. 9). Likewise, both thymic-dependent and -independent development are known for the γ-δTCR$^+$cells (Fig. 9; 1, 9, 78–80, 82). Although our results strongly indicate that DNα-βTCR$^+$ cells expressing NK1.1 are the precursors of IL2-induced LGL or LAK cells, it is not clear whether other DNα-βTCR$^+$ cells are also able to differentiate to LGL upon cultivation in the presence of IL2.

IL2-induced LGL cells also are characterized by the expression of the B220 epitope of the CD45 molecule defined by mAb RA3-6B2. Freshly isolated DNα-βTCR$^+$ and DNγ-δTCR$^+$ cells do not express B220. It is interesting to note that in both cell types, cultivation with IL2 downregulates CD5 but induces the B220 epitope. Although the B220 epitope was originally considered to be a B cell–specific marker (83), evidence has accumulated that this epitope can be expressed not only on B cells but also on T lineage cells upon activation in both α-βTCR$^+$ and γ-δTCR$^+$ cells (30, 84). The CD45 molecule expressing the B220 epitope on such T cells has a different molecular weight from that on B cells (85). DNα-βTCR$^+$B220$^+$ cells are well known to be expanded in autoimmune MLR/*lpr* mice (29, 43, 44). The DNα-βTCR$^+$B220$^+$ population expanded in *lpr/lpr* mice, however, seems to be a different cell type from DNα-βTCR$^+$NK1.1$^+$ cells. The DNα-βTCR$^+$B220$^+$ cells expanded in *lpr/lpr* mice lack IL2Rβ and NK1.1 expression (86, 87). Takeda and Dennert (87) reported an inverse correlation between the level of DNα-βTCR$^+$NK1.1$^+$ cells and the appearance of DNα-βTCR$^+$B220$^+$NK1.1$^-$ cells, which indicates the onset of autoimmunity in *lpr/lpr* mice. It was also shown that the injection of mAb against NK1.1 enhances

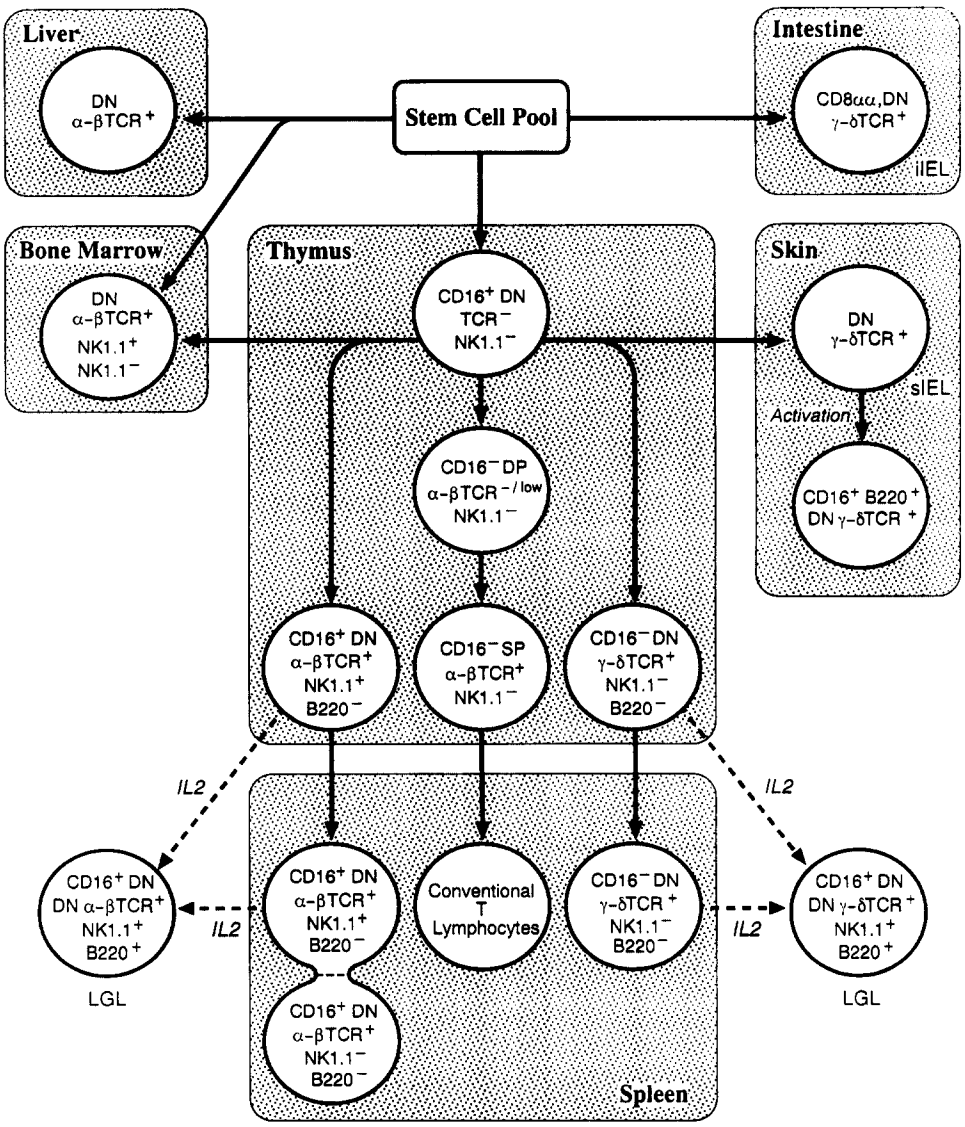

Figure 9. Model for the development of DN LGL cells in adult mice. CD16+DN-TCR−NK1.1− intrathymic precursor cells derived from hematopoietic stem cells differentiate to DP cells expressing a low level of TCR/CD3 (TCR$^{-/low}$) and further differentiate to CD4+ CD8− or CD4−CD8+ SP cells expressing a high level of surface α-βTCR/CD3 complex (1, 71). These conventional SP cells emigrate into peripheral lymphoid organ. In addition to these conventional SP T lymphocytes, DN cells expressing TCR are present in various organs. In thymus, a fraction of thymocytes continues to express both CD16 and the FcεRIγ subunit and differentiate to the DNα-βTCR+CD16+ NK1.1+ thymocytes. These cells emigrate to spleen, lymph node, and bone marrow. In contrast to DNα-βTCR+ cells, DNγ-δTCR+ cells do not express CD16 or NK1.1. Both DNα-βTCR+CD16+NK1.1+ and DNγ-δTCR+CD16−NK1.1− cells in thymus and spleen further differentiate by IL2 stimulation to DN α-β TCR+ CD16+ NK1.1+ B220+ and DNγ-δTCR+CD16+ NK1.1+B220+ LGL, respectively. At least a fraction of DNα-βTCR+ cells in bone marrow and liver develop extrathymically (40, 41). We cannot rule out the possibility that cells which develop extrathymically immigrate to the thymus. DNγ-δTCR+ cells found in the skin (*sIEL*) are derived from thymus and are induced to express CD16 and B220 upon activation (74, 82, 84). γ-δTCR+ cells found in intestine (*iIEL*) develop extrathymically (78–80). It is not known whether all DN-TCR+ cells respond to IL2 and differentiate to LGL. Among the populations shown, DNα-βTCR+CD16+NK1.1+ cells, DNα-βTCR+CD16+NK1.1+B220+ LGL, DNγ-δTCR+CD16+NK1.1+B220+ LGL, and iIEL have been shown to express FcεRIγ as a component of the TCR (this article, and 76, 77).

the appearance of DNα-βTCR+B220+ cells and autoimmunity, whereas adoptive transfer of DNα-βTCR+NK1.1+ cells of normal mice suppressed these symptoms. It is thus likely that DNα-βTCR+B220+ cells expanded in *lpr/lpr* mice are of a distinct origin as compared with DNα-βTCR+NK1.1+ cells and that DNα-βTCR+NK1.1+ cells have an immunosuppressive ability. It is of interest, from this point of view, that DNα-βTCR+NK1.1+ cells in bone marrow also exhibit immunosuppressive activity and seem to function in acute bone marrow graft rejection (38, 39).

DNα-βTCR+ thymocytes lack CD4 and CD8, which are important in thymic selection. Indeed, the V gene repertoire of DNα-βTCR+ thymocytes suggests that this population does not undergo normal thymic selection pathways and contains autoreactive cells (88, 89). This is possibly because of the lack of CD4/CD8 expression. Alternatively, it is possible that the TCRs containing FcεRIγ subunits transmit distinct signals when interacting with thymic MHC molecules resulting in different selection mechanisms. From this point of view, it is of interest that CD3ζ/η, FcεRIγ, and other CD3 subunits contain an amino acid sequence motif originally pointed out by Reth (YxxLxxxxxxxYxxL) (90). Whereas this motif is repeated three times in CD3ζ and twice in CD3η, FcεRIγ has only one motif with different surrounding sequences. Studies with chimeric molecules consisting of the intracellular portion of proteins containing this

motif connected with an extracellular domain of an unrelated receptor molecule show the importance of this motif and qualitative differences in signal transduction between motifs derived from distinct molecules (91–97). Key signal transduction molecules such as $p56^{lyn}$ and/or ZAP70 may be differentially associated with distinct TCR isoforms and transmit different signals. In this context, it is of note that in LGL, an elevation of the intracellular cAMP level, but not a combination of Ca^{2+} mobilization and activation of protein kinase C, induces expression of the IL2Rα subunit (46) in contrast to the findings in conventional T lymphocytes (98).

In summary, we have demonstrated that DN-TCR$^+$ CD16$^+$NK1.1$^+$B220$^+$CD5$^-$ LGL cells are induced by IL2 from both DNα-βTCR$^+$CD16$^+$NK1.1$^+$B220$^-$CD5$^+$ and DNγ-δTCR$^+$CD16$^-$NK1.1$^-$B220$^-$CD5$^+$ cells present in thymus and spleen. FcϵRIγ is expressed in freshly isolated DNα-βTCR$^+$CD16$^+$NK1.1$^+$B220$^-$CD5$^+$ but not in DNγ-δTCR$^+$CD16$^-$NK1.1$^-$B220$^-$CD5$^+$ thymocytes. Incubation of these cells with IL2 greatly induces the expression of FcϵRIγ and the TCR contains FcϵRIγ as a subunit in the resulting LGL. It is now critical to ascertain the function of distinct CD3ζ/η-FcϵRIγ dimers in signal transduction and in the development of the DNα-βTCR$^+$ population.

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