

Elimination In Vivo of Developing T Cells by Natural Killer Cells

Eckart Schott, Roberto Bonasio, and Hidde L. Ploegh

Department of Pathology, Harvard Medical School, Boston, MA 02115

Abstract

Natural killer cells gauge the absence of self class I MHC on susceptible target cells by means of inhibitory receptors such as members of the Ly49 family. To initiate killing by natural killer cells, a lack of inhibitory signals must be accompanied by the presence of activating ligands on the target cell. Although natural killer cell-mediated rejection of class I MHC-deficient bone marrow (BM) grafts is a matter of record, little is known about the targeting in vivo of specific cellular subsets by natural killer cells. We show here that development of class I MHC-negative thymocytes is delayed as a result of natural killer cell toxicity after grafting of a class I MHC-positive host with class I MHC-negative BM. Double positive thymocytes that persist in the presence of natural killer cells display an unusual T cell receptor-deficient phenotype, yet nevertheless give rise to single positive thymocytes and yield mature class I MHC-deficient lymphocytes that accumulate in the class I MHC-positive host. The resulting class I MHC-deficient CD8 T cells are functional and upon activation remain susceptible to natural killer cell toxicity in vivo. Reconstitution of class I MHC-deficient BM precursors with H2-K^b by retroviral transduction fully restores normal thymic development.

Key words: thymocyte development • bone marrow chimera • NK cell toxicity • CD8 T cells • retroviral infection

Introduction

NK cells are BM-derived lymphocytes that are active in the defense against tumors and virally infected cells. NK cells also play a role in rejection of transplants of hematopoietic origin that fail to express the full range of host self class I MHC molecules (1) or that lack class I MHC molecules altogether (2). However, the rejection of BM may not be complete or may selectively affect specific cellular subsets.

Activation of NK cells is tightly controlled by a variety of inhibitory and activating receptors (3). The murine Ly49 molecules are polymorphic members of the C-type lectin family expressed on NK cells, where they act as receptors for specific class I MHC alleles (for reviews see references 4, 5). A target cell that lacks expression of class I MHC molecules can activate NK cells, presumably by failing to engage the appropriate inhibitory receptor on the NK cell (6–10). In general, more than one Ly49 marker is expressed on any given NK cell, often in monoallelic fashion. Engagement of Ly49 leads to suppression of NK cell function through ITIM-dependent recruitment of the phosphatase SHP-1 (11). The activation

threshold of an NK cell with a given Ly49 repertoire is further controlled by fine tuning in response to expression levels of class I MHC alleles of the host in which the NK cell arises (12–14). Repeated contact of a Ly49 molecule with the appropriate self-ligands leads to its down-regulation, thereby tuning the sensitivity of NK cells. Therefore, the ability of NK cells to eradicate BM grafts with reduced levels of class I MHC is calibrated on the levels of class I MHC in the host in which NK cells are educated: class I MHC-deficient NK cells no longer kill untransformed class I MHC-deficient target cells (15, 16).

NKG2 molecules constitute another class of NK cell receptors and are also members of the C-type selectin superfamily. They heterodimerize with CD94 to form inhibitory (NKG2A) or activating receptors (NKG2C, E) or homodimerize to form the activating NK cell receptor NKG2D (for review see reference 17). The class Ib MHC molecule Qa1, loaded with a peptide derived from the signal sequence of H2-D molecules (18), is a ligand for NKG2A, C, E (19, 20), whereas RAE-1 (21) and H-60 (22), molecules with

Address correspondence to Hidde L. Ploegh, Dept. of Pathology, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-4777; Fax: (617) 432-4775; email: ploegh@hms.harvard.edu

Abbreviations used in this paper: CFSE, 5,6-carboxyfluorescein-diacetate-succinimidyl-ester; DN, double negative; DP, double positive; lin, lineage; SP, single positive.

some structural similarity to class I MHC molecules, are ligands for NKG2D (23, 24).

The missing self-hypothesis postulates inhibition of a “tonic” level of NK cell toxicity by the expression of self class I MHC ligands and adequately explains the majority of NK cell functions. However, others such as the hybrid histocompatibility phenomenon (1) predict the presence of activating signals that can override inhibitory signals. It follows that certain normal cell types, including thymocytes, must express yet to be defined activating ligands whose binding to NK cell receptors can elicit killing, at least in the absence of inhibitory signals (25–27). Although the identity of such activating ligands remains poorly understood, triggering of NK cells in experimental models is facilitated when examining transformed or activated target cells: class I MHC-deficient NK cells can target class I MHC-deficient tumor cells but not class I MHC-deficient ConA blasts (28, 29). Targeting of B cell lines by NK cells *in vitro* is highly dependent on the state of differentiation and activation of the target cell (30).

We have shown earlier that functional CD8 T cells are generated from $K^bD^b^{-/-}$ BM grafts transferred into class I MHC competent $RAG^{-/-}$ hosts (31, 32). In the course of these experiments, we observed a striking developmental anomaly in the generation of such T cells, possibly attributable to NK cell-mediated killing of developing T cells.

Thymocytes are generated from common lymphocyte precursors that migrate to the thymus and give rise to $CD4^-CD8^-$ double negative (DN) thymocytes (for review see reference 33). During the DN phase of thymocyte development, rearrangement of the TCR β chain occurs, and a pre-TCR complex consisting of TCR β and the invariant pT α chain is expressed as the result of a process termed β selection (for review see reference 34). The pre-TCR complex drives rapid proliferation of the thymocytes that express it. Down-regulation of CD25 is achieved during this rapid proliferative phase (35). Failure to express the pre-TCR complex blocks the transition from the DN to the $CD4^+CD8^+$ double positive (DP) stage, where rearrangement of the TCR α chain occurs. The resulting TCR $\alpha\beta$ complex is the entity that mediates positive and negative selection (for reviewed see reference 36). In the absence of pT α , DN thymocytes are arrested at the $CD44^-CD25^+$ stage (DN3) and lack TCR β expression (37). Few cells overcome this block, and rather large numbers of $\gamma\delta$ T cells result from such precursors.

By engrafting a class I MHC-positive host with class I MHC-deficient BM, we observe accumulation of T cells in the host, despite the presence of NK cells that should presumably recognize the class I MHC-deficient graft. *In vivo* depletion of the majority of thymocytes is NK cell dependent and reveals a population of TCR-negative DP thymocytes that reach this stage without rapid cell division and by circumventing β selection. The mature single positive (SP) CD8 T cells that develop from such precursors are exported to the periphery and upon activation remain fully susceptible to NK cell killing *in vivo*.

Materials and Methods

Animals, BM Transfer, and Surgical Procedures. C57/BL6J ($H2^b$), C57/BL6J $RAG-1^{-/-}$, C57/BL6J $TAP^{-/-}$, and C57/BL6J $\beta 2m^{-/-}$ mice were purchased from Jackson Laboratories. $RAG-2^{-/-}$ common gamma chain (γc) $^{-/-}$ mice and C57/BL6J perforin $^{-/-}$ mice were obtained from Taconic. $K^bD^b^{-/-}$ mice have been described (38); they were backcrossed 10 times to C57/BL6J littermates and then intercrossed to obtain $K^bD^b^{-/-}$ animals. OT-I $K^bD^b^{-/-}$ mice were generated by breeding OT-I mice (39) to $K^bD^b^{-/-}$ mice. All mice were kept under pathogen-free conditions in the HMS animal facility. Phenotyping was performed by staining of peripheral blood lymphocytes with fluorescently labeled antibodies against V $\alpha 2$, V $\beta 5$, CD8, B220, H2-K b , and H2-D b (all from Becton Dickinson) followed by flow cytometry.

BM cells were harvested from femur and tibia under sterile conditions, and erythrocytes were lysed. T lymphocytes were depleted using THY1.2-coated magnetic beads and MACS-LD columns (Miltenyi), and 10^7 cells were transferred into sublethally irradiated (600 rad) $RAG-1^{-/-}$ mice. Recipients were fed trimethoprim/sulfamethoxazol with the drinking water and were killed at the times indicated.

Depletion of NK cells was effectuated by weekly *i.p.* injection of 25 μg anti-NK1.1 antibody, starting on the day before transfer of the BM graft or of peripheral CD8 T cells. Control mice were injected with the appropriate isotype control antibody (both from eBioscience).

Thymic transplants were performed according to standard procedures. In brief, thymic lobes were removed from chimeric mice 3 wk after transfer of OT-I $K^bD^b^{-/-}$ BM, *i.e.*, at a time point at which no mature DP or SP cells were present. Thymic lobes were transplanted under the kidney capsule of $RAG^{-/-}$ $\gamma c^{-/-}$ mice. Recipient mice were killed 5 wk after the transplantation, and thymic grafts and spleens were removed and analyzed by flow cytometry.

Flow Cytometry. Single cell suspensions were generated from BM, thymus, and spleen, and erythrocytes were lysed. Cells were washed in FACS[®] buffer (PBS, 0.5% wt/vol BSA, 0.02% wt/vol Na-Azide) and stained with FITC-anti-TCR β , PE-anti-V $\alpha 2$, FITC-anti-V $\beta 5$, PE-anti-CD4, CyC-anti-CD8, CyC-anti-CD44, FITC-anti-CD25, CyC-anti-B220, PE-anti-K b , PE-anti-D b , PE-anti-NK1.1, PE-anti-fas, FITC-anti-c-kit, and biotin-anti-sca-1 followed by streptavidin-CyC. The lineage markers (lin) comprised PE-conjugated antibodies against CD3 ϵ , CD19, NK1.1, IgM, Gr-1, and Ter-119. All antibodies were obtained from Becton Dickinson or eBioscience. Samples were washed twice in FACS[®] buffer before analysis on a Becton Dickinson FACScalibur using CellQuest software.

Screening for V β chains was performed with the mouse V β TCR screening panel from Becton Dickinson. For intracellular staining, cells were first stained for surface markers, followed by fixation in 0.5% paraformaldehyde and permeabilization in 0.5% saponin (Sigma-Aldrich). Staining was then performed with CyC-anti-TCR β or APC-anti-IFN γ (Becton Dickinson) in buffer containing 0.5% saponin on ice. Cells were washed in 0.5% saponin twice before resuspension in FACS[®] buffer and analysis.

K^b (SIINFEKL) tetramers were produced as described (40). In brief, recombinant H2-K b and $\beta 2m$ were refolded in the presence of SIINFEKL peptide. The resulting monomers were biotinylated *in vitro*, purified by gel filtration, and tetramerized with PE-labeled streptavidin. The resulting tetramers were again purified by gel filtration and yielded a single peak of appropriate size.

Bromodeoxyuridine Labeling. Chimeric mice were generated as described above. 4 wk after the BM transfer, mice were injected with 1 mg/mouse of bromodeoxyuridine (BrdU; Sigma-Aldrich) in PBS at 0 and 4 h. Mice were killed at 24 h, and thymi were removed. After preparation of single cell suspensions, cells were stained for surface markers and then fixed in 95% ethanol, followed by 1% paraformaldehyde/0.01% Tween20 (Sigma-Aldrich)

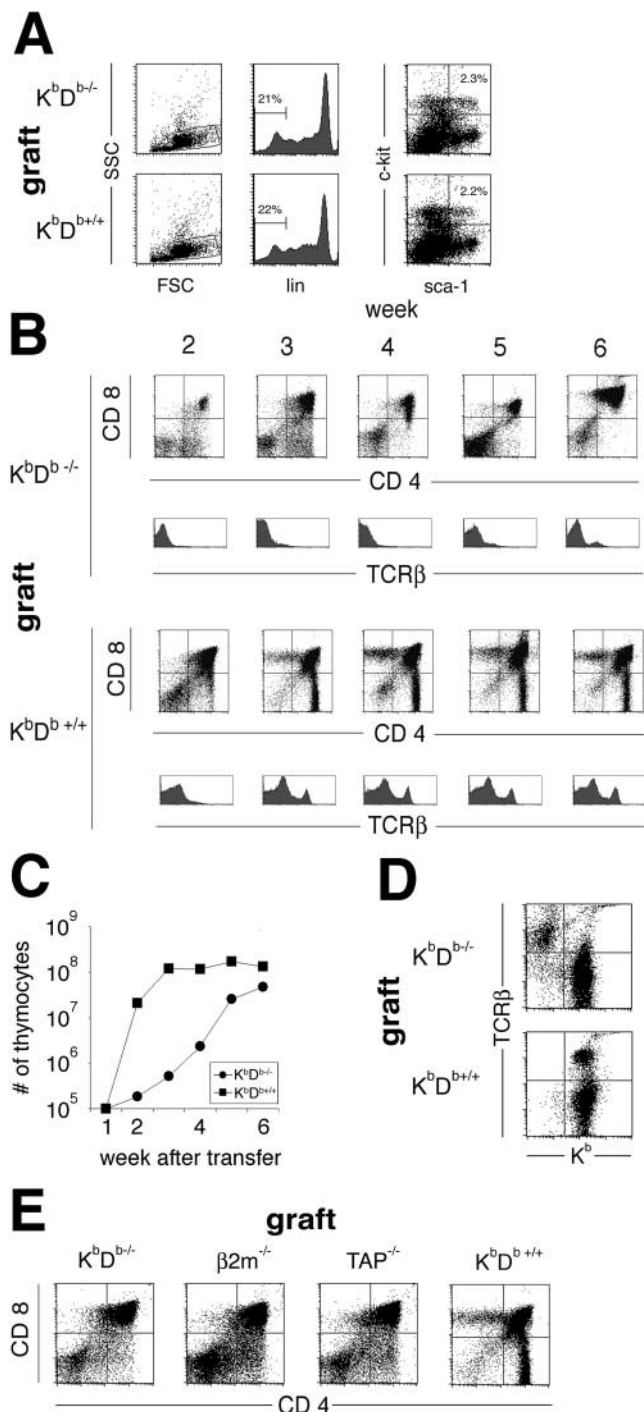


Figure 1. Generation of DP and SP thymocytes from $K^bD^b^{-/-}$ BM grafts is delayed. (A) BM from $K^bD^b^{-/-}$ and $K^bD^b^{+/+}$ mice was stained with lineage marker antibodies (lin) and with antibodies against c-kit and

and DNase treatment (50 Kunitz U/ml) as described (41). BrdU incorporation was detected by staining with FITC-anti-BrdU (1 μ g/ 10^6 cells; eBioscience), in the presence of 0.5% Tween20, followed by flow cytometry.

Retroviral Infection of BM Cells. The H2-K^b and H2-D^b cDNAs were amplified by RT-PCR from mRNA isolated from B6 splenocytes with Superscript II reverse transcriptase and Pfx polymerase (both from Invitrogen) using the appropriate primers to introduce a 5' XhoI (K^b) or EcoRI (D^b) and a 3' EcoRI site into the PCR product. The fragments were subcloned into pSP-72 (Promega), and their sequences were confirmed by sequencing using the SP6 promoter and T7 promoter primers. Both fragments were subcloned into the XhoI and EcoRI sites of the vector MIG-R, encoding a modified version of mouse stem cell virus that includes an IRES-driven EGFP and an RNA export signal (42). The constructs will henceforth be referred to as MIG, MIG-K^b, and MIG-D^b.

Virus was propagated by transfecting the constructs MIG, MIG-K^b, and MIG-D^b into 293 cells stably transfected with GAG, POL, and VSV-G under a tetracycline-inducible silencer (43). Culturing the cells in media without tetracycline resulted in generation of VSV-G pseudotyped virus, which was concentrated by centrifugation (16,500 rpm for 3 h).

Donor mice were treated with 5 mg/mouse of 5-fluorouracil (Sigma-Aldrich) i.p. 5 d before the harvest of BM. BM cells were harvested (day 0) and cultured at 10^6 cells/ml in IMDM supplemented with 10% FCS, 50 μ g/ml β -mercaptoethanol, penicillin/streptomycin, 25 ng/ml IL-6, 25 ng/ml IL-7, 50 ng/ml CSF, and 50 ng/ml flt-3-ligand (all from R&D systems). Cells were infected on days 2 and 4 by centrifugation with concentrated virus supernatant at 2,200 rpm, 30°C, for 1.5 h, in the presence of 8 μ g/ml polybrene (Sigma-Aldrich).

After analysis of the infection efficiency (EGFP⁺ cells) and expression of lineage markers by flow cytometry, equal numbers of EGFP⁺ lin⁻ cells were transferred i.v. into irradiated RAG^{-/-} recipients on day 5. Mice were killed after 4 wk, and thymi and spleens were harvested and analyzed by flow cytometry.

Carboxyfluorescein-diacetate-succinimidyl-ester Labeling of Peripheral CD8 T Cells. $K^bD^b^{-/-}$ OT-I CD8 T cells were generated as described (32). In brief, OT-I $K^bD^b^{-/-}$ BM was transferred into sublethally (600 rad) irradiated RAG^{-/-} hosts. LNs were harvested 10 wk later. $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ OT-I CD8 T cells were isolated from LNs and purified using the CD8 T cell purification kit from Miltenyi. Cells were labeled in vitro with 5,6-carboxyfluorescein-diacetate-succinimidyl-ester (CFSE; Molecular Probes) (15 μ M, 10 min, 37°C) and equal numbers were transferred i.v. into the indicated hosts. Mice were stimulated with antigen by i.p. injection of 20 μ g SIINFEKL peptide in PBS 24 h after transfer. Another 48 h later, mice were killed, and LNs and spleens were harvested. Cells were analyzed by flow cytometry after staining with PE-K^b(SIINFEKL)-tetramer and CyC-anti-

sca-1 to establish the frequency of progenitor cells. (B) BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated RAG^{-/-} hosts. Mice were killed at the times indicated, and thymi were analyzed by flow cytometry after staining for CD4, CD8, and intracellular TCR β . (C) The total number of thymocytes retrieved at different time points after transfer of $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ BM is indicated. (D) Spleen cells from chimeras that had received $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ BM were analyzed by flow cytometry 10 wk after BM transfer. (E) BM from $K^bD^b^{-/-}$, $\beta 2m^{-/-}$, TAP^{-/-}, or $K^bD^b^{+/+}$ (B6) mice was transferred into sublethally irradiated RAG^{-/-} hosts. Thymi were analyzed at 4 wk by flow cytometry after staining for CD4 and CD8.

CD8 α . Total numbers of cells retrieved from spleen and LNs were determined.

Results

Development of DP and SP Thymocytes Is Delayed in the Absence of Class I MHC Molecules on Thymocytes. To assess the consequence of lack of class I MHC molecules on thymocyte development, we made use of an adoptive transfer model in which synchronized thymic development is achieved. Equal numbers of T cell–depleted BM cells from K^bD^{b-/-} or K^bD^{b+/+} C57/BL6J (H-2^b) mice were transferred into sublethally irradiated C57/BL6J RAG^{-/-} recipients. Staining of BM from both donors confirmed the presence of similar numbers of lin⁻ cells and c-kit/sca-1 DP cells in the lin⁻ compartment of both donor populations (Fig. 1 A). Thymi and spleens of recipient mice were analyzed at 2–8 wk after transfer. Thymic profiles of mice that had received K^bD^{b-/-} stem cells showed a pronounced delay in development of SP thymocytes compared with mice that received K^bD^{b+/+} stem cells (Fig. 1 B). No SP thymocytes were detected until week 5, whereas such cells appeared in thymi of K^bD^{b+/+} grafted mice as early as 2 wk after transfer. The defect applied to both CD4 and CD8 SP thymocytes. Between week 6 and 8, depending on the individual experiment, SP thymocytes emerged in the thymi of K^bD^{b-/-}-grafted animals. The total number of thymocytes accumulating in the thymus was reduced, and the kinetics of their generation was slowed (Fig. 1 C). The delay in thymic development was mirrored by a similarly delayed appearance of SP lymphocytes in the periphery, as detected by staining of splenocytes for CD4 and CD8 (not depicted). However, mature T cells accumulated in the periphery at 8 to 10 wk (Fig. 1 D).

The observed effect is not an artifact related to the targeted mutations harbored by K^bD^{b-/-} mice, since a similar delay in development was observed after transfer of BM from mice with other mutations that affect class I MHC expression namely TAP^{-/-} and β 2m^{-/-} mice (Fig. 1 E). Although in TAP^{-/-} and β 2m^{-/-} mice low levels of class I MHC are expressed on the cell surface, such expression was not sufficient to restore normal patterns of T cell development in the thymus. As shown in Fig. 6, normal T cell development could be restored by retroviral transduction of a functional H2-K^b construct into the class I MHC–deficient BM graft. These results either suggest a cell autonomous role of class I MHC molecules in the development of T cells or can be accounted for by the ability of NK cells to recognize and eliminate class I MHC–deficient cells.

NK Cell Toxicity Is Responsible for the Loss of DP and SP Thymocytes. To address the question whether an NK cell response is responsible for the observed phenotype, recipient RAG^{-/-} mice were treated with NK1.1 antibody to deplete NK cells in vivo or with the appropriate isotype control antibody. Transfer of K^bD^{b-/-} BM into NK cell–depleted mice resulted in complete restoration of the patterns of thymocyte development as observed after transfer of class I MHC–positive grafts (Fig. 2 A). To establish the

mechanism by which NK cell exert this effect, we transferred class I MHC–positive or –negative BM from OT-I RAG^{-/-} mice into C57/BL6 perforin^{-/-} mice. The OT-I TCR is specific for SIINFEKL presented by H2-K^b. BM from transgenic animals was chosen to distinguish thymocytes derived from the graft from endogenous thymocytes that are present, since the host animals were RAG competent. Analysis of the patterns of thymocyte development showed that transfer of K^bD^{b-/-} BM into perforin-deficient hosts allowed normal generation of SP OT-I CD8 T cells as detected by K^b-SIINFEKL tetramer staining (Fig. 2 B).

Although accumulation of NK cells was observed in thymus and spleen of mice grafted with K^bD^{b-/-} BM, staining of infiltrating NK cells for intracellular IFN γ content showed little IFN γ production (Fig. 2 C). Since it has been proposed that fas–fasL–dependent killing might be a mechanism by which NK cells exert cytotoxicity (44–46), we stained thymocytes from K^bD^{b-/-} and K^bD^{b+/+} grafts with anti-fas antibodies. Levels of fas surface expression were not significantly different in DP thymocytes from class I MHC–positive or –negative grafts (Fig. 2 D). Although we cannot exclude that additional mechanisms are involved in the NK cell–mediated toxicity in this model, the reason for the selective killing of K^bD^{b-/-} thymocytes is unlikely to be due solely to differential expression of fas.

DP Thymocytes Generated from Class I MHC Grafts in the Presence of NK Cells Display an Abnormal Phenotype. Even though class I MHC–deficient grafts are rejected in an NK cell–dependent fashion, we readily detected the presence of class I MHC–deficient DP thymocytes in hosts engrafted with class I MHC–deficient BM. What are the properties of these cells? DP thymocytes generated from class I MHC–deficient stem cells showed no TCR β expression in the early course of thymic development, as judged from intracellular and surface staining for TCR β (Fig. 3 A). Although they up-regulated TCR β expression later on, it is striking that these DP thymocytes are present at all. They must have evaded the checkpoint controlled by the pre-TCR that usually prevents thymocytes from progressing to the DP stage in the absence of productive TCR β rearrangement and TCR β expression. Another phenotypic characteristic of DP thymocytes generated from class I MHC–deficient stem cells is the failure to down-regulate CD25, which usually occurs during the rapid proliferation phase of pT α –positive cells (35).

The failure of class I MHC–deficient DP thymocytes to down-regulate CD25 expression suggests a defect in rapid proliferation of DN thymocytes, similar to what is observed in mice that do not express pre-TCR α (pT α) (37). We hypothesized that early expression of a transgenic TCR should rescue thymic development if insufficient pT α expression and β selection were responsible for the observed developmental delay (47). To that end, OT-I transgenic K^bD^{b-/-} mice were analyzed. The transfer of OT-I K^bD^{b-/-} stem cells resulted in a similar developmental delay of CD8 T cells as that seen for nontransgenic stem cells (Fig. 3 B). Although high levels of OT-I TCR

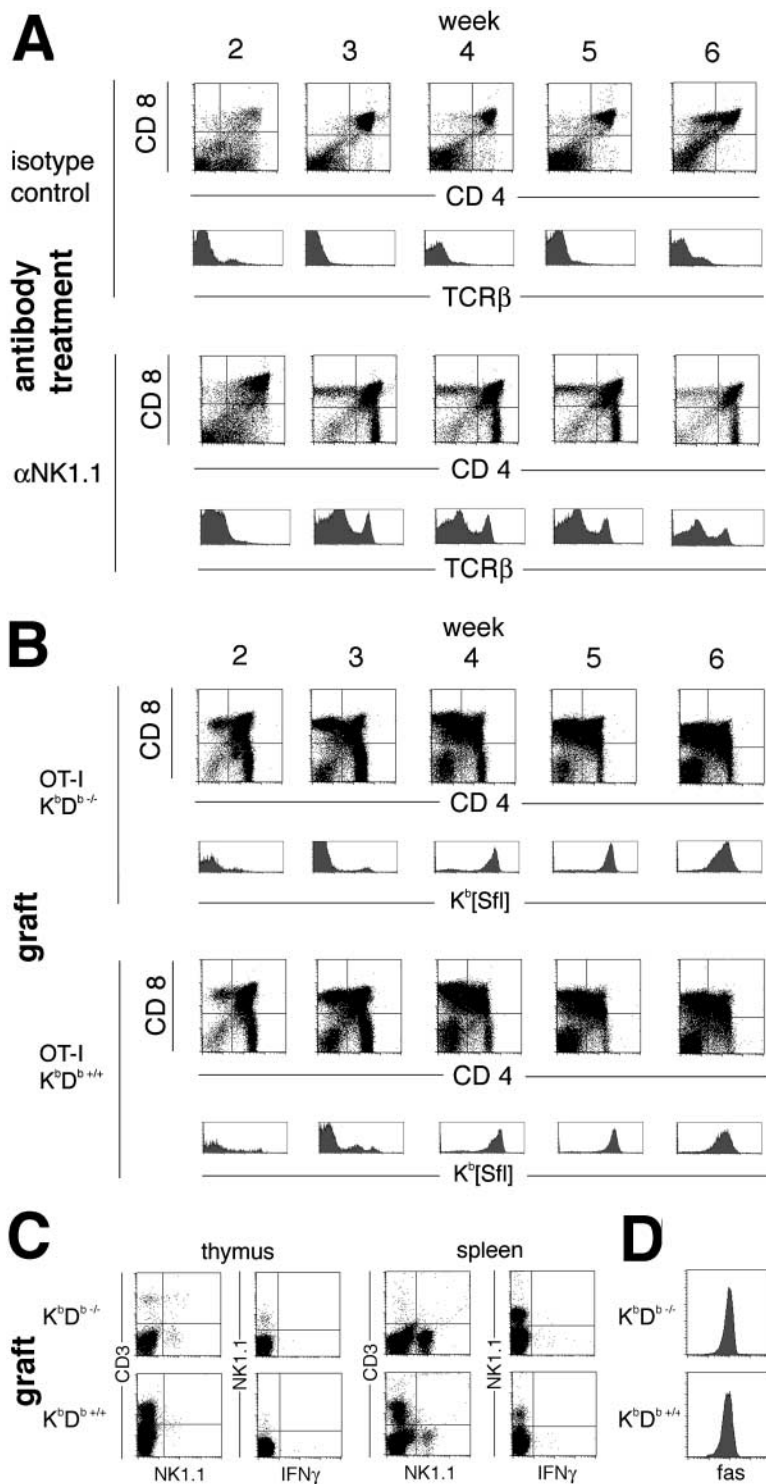


Figure 2. The delay in development of $K^bD^b^{-/-}$ thymocytes is due to NK cell toxicity. (A) BM from $K^bD^b^{-/-}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts that were treated with NK1.1 antibody or the appropriate isotype control. Mice were killed at the times indicated, and thymi were analyzed by flow cytometry after surface staining for CD4, CD8, and intracellular TCR β . (B) BM from OT-I $K^bD^b^{-/-}$ or OT-I $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated perforin $^{-/-}$ hosts. Mice were killed at the times indicated, and thymi were analyzed by flow cytometry after surface staining for CD4 and CD8. Since endogenous thymocytes are present, the presence of thymocytes expressing the transgenic TCR OT-I was visualized by staining with K^b (SIINFEKL) tetramer. (C) BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. Mice were killed at 4 wk, and thymi and spleens were analyzed by flow cytometry after surface staining for CD3 and NK1.1, and intracellular staining for IFN γ . (D) Experimental setup as in C, but staining was performed with anti-CD4, anti-CD8, and anti-fas antibodies. Data shown in the histograms result from gating on DP thymocytes.

were readily detected in the vast majority of thymocytes derived from OT-I $K^bD^b^{+/+}$ stem cells as early as 2 wk after transfer, no such expression was found in the thymocytes derived from their OT-I $K^bD^b^{-/-}$ counterparts. Also, OT-I $K^bD^b^{-/-}$ thymocytes showed the same defect in down-regulating the expression of CD25 (not depicted) as seen in the nontransgenic $K^bD^b^{-/-}$ grafts. Al-

though the potency of a TCR $\alpha\beta$ to drive the DN to DP transformation is much reduced compared with a pre-TCR (48), the failure to restore a normal phenotype of DP thymocytes by expression of a TCR $\alpha\beta$ argues against a role for pT α as the underlying mechanism. Also, no increased numbers of TCR $\gamma\delta$ -expressing thymocytes were observed (not depicted).

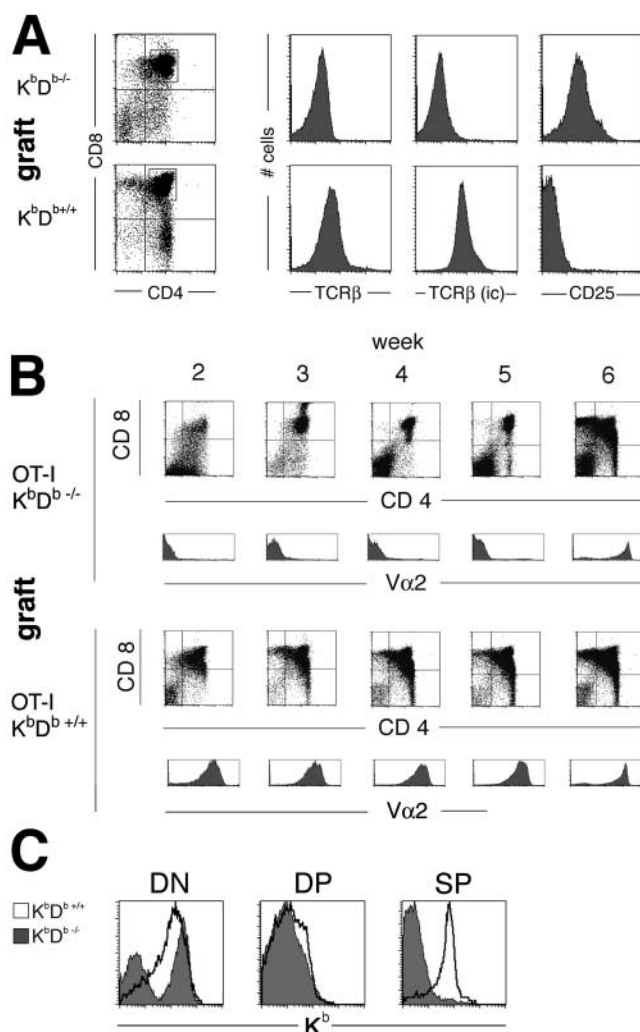


Figure 3. $K^bD^b^{-/-}$ DP thymocytes developing in the presence of NK cells display an immature phenotype. (A) BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. Mice were killed at 4 wk, and thymi were analyzed by flow cytometry after surface staining for CD4, CD8, TCR β , CD25, and intracellular staining for TCR β . Data shown in the histograms results from gating on DP thymocytes as shown in the left panels. (B) BM from OT-I $K^bD^b^{-/-}$ or OT-I $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. Mice were killed at the times indicated, and thymi were analyzed by flow cytometry after surface staining for CD4, CD8, and with K^b(SIINFEKL) tetramer to visualize presence of the OT-I TCR. (C) Experimental setup as in A, but staining was performed with anti-CD4, anti-CD8, and anti-H2-K^b antibodies in chimeras 8 wk after the transfer of BM. Histograms show data resulting from gating on DN, DP, or CD8 SP thymocytes.

We analyzed the normal course of expression of class I MHC in developing thymocytes. Although high levels of H2-K^b were detected in DN and SP thymocyte populations of $K^bD^b^{+/+}$ grafted thymi, almost complete down-regulation of H2-K^b was found in DP thymocytes, resembling staining patterns of $K^bD^b^{-/-}$ thymocytes (Fig. 3 C). The H2-K^b-positive population seen in DN thymocytes from $K^bD^b^{-/-}$ -grafted hosts are host derived. Down-regulation of class I MHC is a normal trait in thymocyte development (49). The selective susceptibility of class I MHC-

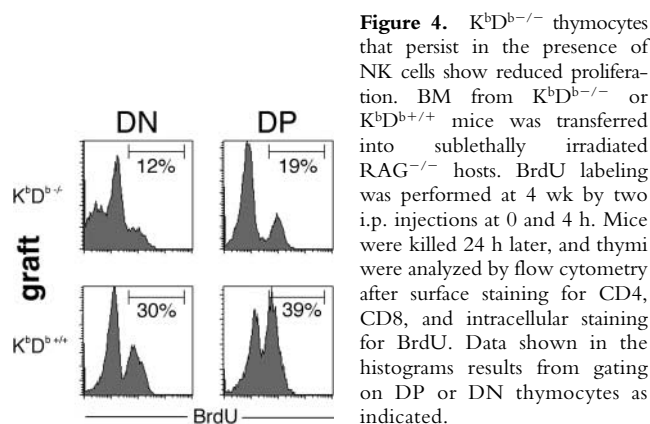


Figure 4. $K^bD^b^{-/-}$ thymocytes that persist in the presence of NK cells show reduced proliferation. BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. BrdU labeling was performed at 4 wk by two i.p. injections at 0 and 4 h. Mice were killed 24 h later, and thymi were analyzed by flow cytometry after surface staining for CD4, CD8, and intracellular staining for BrdU. Data shown in the histograms results from gating on DP or DN thymocytes as indicated.

deficient grafts to NK cell killing therefore likely results from differential expression of a surface marker that correlates with activation status or the proliferative activity of a thymocyte. To that end, we investigated whether the DP thymocytes generated from $K^bD^b^{-/-}$ grafts result from a pathway of maturation that involves less proliferative activity than is the case for regular $K^bD^b^{+/+}$ DP thymocytes.

$K^bD^b^{-/-}$ Thymocytes that Persist in the Presence of NK Cells Show Reduced Proliferation. The much reduced number of thymocytes and the delayed kinetics of thymocyte accumulation suggest a defect in initiating proliferation of DN thymocytes. Alternatively, NK cells could selectively attack proliferating cells that express NK-activating ligands. Only thymocytes that display reduced levels of such activating ligands, for example, because they proliferate less rapidly, would be spared deletion by NK cells. Therefore, we analyzed the proliferation profiles of thymocytes 4 wk after transfer of the BM graft by BrdU labeling. Mice were labeled with BrdU by i.p. injection at 0 and 4 h. Thymi were removed and analyzed at 24 h. Compared with thymi engrafted with $K^bD^b^{+/+}$ progenitors, we observed reduced numbers of cells that proliferated during the pulse period in the DN and DP compartments of thymi grafted with $K^bD^b^{-/-}$ progenitors (2.5-fold less for DN cells and 2.1-fold less for DP cells; Fig. 4). This result, together with the failure of $K^bD^b^{-/-}$ grafts to down-regulate CD25 and express TCR β , suggests that the DP thymocytes generated from a $K^bD^b^{-/-}$ graft in the presence of NK cells develop along an alternative pathway that does not involve rapid proliferation and efficient TCR β rearrangement. In contrast to cells that are targeted by NK cells, the DP thymocytes produced by this alternate pathway, characterized by reduced proliferation, appear much less susceptible to NK cell killing.

Phenotypically Distinct Thymocytes Generated in the Presence of NK Cells Give Rise To Polyclonal Mature CD8 T Cells. To rule out the possibility that the mature T cells that accumulated in the chimeras grafted with $K^bD^b^{-/-}$ BM are derived from a single clone of progenitor cells that has acquired resistance to NK cell toxicity, we analyzed the expression of V β chains in peripheral T cells. To that end, cells were harvested from the spleen 8 wk after BM transfer

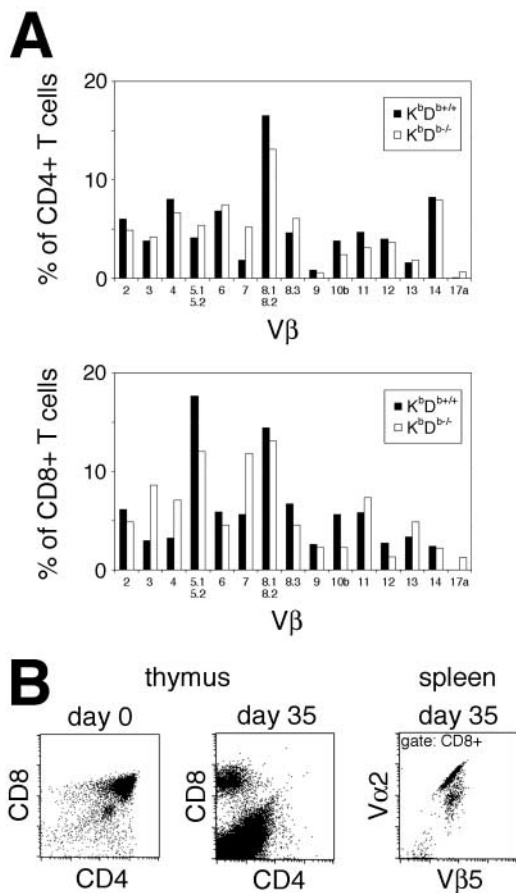


Figure 5. Phenotypically distinct $K^bD^b^{-/-}$ thymocytes generated in the presence of NK cells give rise to polyclonal mature T cells. (A) BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. Mice were killed at 10 wk, and spleens were analyzed by flow cytometry after surface staining with anti-CD4 and anti-CD8 antibodies and with a panel of different anti-V β antibodies. The percentage of spleen cells staining with each individual anti-V β antibody is shown for animals grafted with $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ BM. (B) BM from $K^bD^b^{-/-}$ or $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. Mice were killed at 3 wk, and one thymic lobe was analyzed by flow cytometry after surface staining for CD4 and CD8. The corresponding thymic lobe was transplanted under the kidney capsule of $RAG^{-/-} c\gamma c^{-/-}$ mice. Recipient mice were killed 5 wk after the procedure, and thymic grafts and spleens were analyzed by flow cytometry after staining with anti-CD4, anti-CD8, anti-V α 2, and anti-V β 5 antibodies.

and stained with a panel of V β antibodies. Although slight differences in the frequency of expression were noted for the V β chains tested, multiple V β chains were detected and clearly demonstrate the polyclonal origin of CD4 and CD8 T cells generated from $K^bD^b^{-/-}$ grafts in the presence of NK cells (Fig. 5 A).

We next examined whether SP thymocytes were generated from the phenotypically distinct DP thymocyte population and not from newly arising phenotypically normal DP thymocytes. To that end, we transplanted thymic lobes from chimeras grafted with OT-I $K^bD^b^{-/-}$ BM into $RAG^{-/-} c\gamma c^{-/-}$ animals 3 wk after the initial BM transfer. The complete absence of NK cells in $c\gamma c^{-/-}$ mice ensured that newly generated T cells were not eradicated by NK

cells. At the time of transplantation, no mature DP or SP thymocytes were observed in the grafts (Fig. 5 B). 5 wk after the transplantation, spleen and thymus were removed from recipients of the thymic grafts and analyzed for the presence of transgenic CD8 T cells by staining with anti-V α 2 and anti-V β 5 antibodies. Both thymic graft and spleen contained abundant OT-I CD8 T cells (Fig. 5 B). The presence of such cells suggests that mature SP CD8 T cells are generated from the phenotypically distinct DP thymocyte population rather than from newly arising phenotypically normal DP thymocytes. We cannot formally exclude the possibility that the few DN cells present in the thymus at the time of transplantation generate the CD8 SP cells.

Reconstitution of Class I MHC-deficient BM Stem Cells with H2-K^b Rescues Early Development of Thymocytes. Our data show that class I MHC molecules play a crucial role in early lymphocyte development in the presence of NK cells. Reconstitution of $K^bD^b^{-/-}$ BM with class I MHC molecules should restore normal generation of DP and SP thymocytes. We infected $K^bD^b^{-/-}$ BM in vitro with a retrovirus that encodes full-length H2-K^b or H2-D^b cDNAs and an IRES that drives expression of EGFP, or with a retrovirus that encodes EGFP alone. Infection efficiencies ranged from 45 to 65% as judged from the frequency of EGFP⁺ cells. On day 5 of culture, BM cells were analyzed for expression of lineage markers and EGFP (Fig. 6 A) and expression of the respective class I MHC molecule. Equal numbers of EGFP⁺ lin^{-} cells were then transferred i.v. into recipient $RAG^{-/-}$ mice. Mice were killed and analyzed 4 wk after the transfer.

Analysis of the spleens of the different chimeras showed that BM infected with MIG-K^b or MIG-D^b gave rise to EGFP-expressing B cells, whereas no such B cells were observed in animals that had received MIG-infected class I MHC-deficient BM (Fig. 6 B), establishing that chimeras were successfully generated from BM infected with MIG-K^b and MIG-D^b. Retroviral transduction obviously confers a disadvantage on the grafted cells, explaining why mice that received BM infected with the vector expressing only EGFP showed reconstitution mostly by noninfected stem cells. The BM of such animals also contained only few transduced cells, as determined by the number of EGFP⁺ cells (not depicted). Expression of H2-K^b in BM grafts resulted in rescue of thymocyte development, unlike grafts transduced with only the empty vector (Fig. 6 C). Because the injected cells consist of a mixture of retrovirally infected and noninfected stem cells, each mouse contains a population of cells that serves as an internal control. The comparison of transduced (EGFP⁺) and nontransduced (EGFP⁻) thymocytes showed that H2-K^b-positive cells matured normally in the thymus, down-regulated CD25, and up-regulated TCR β , whereas nontransduced (EGFP⁻) thymocytes displayed the immature phenotype described above. In contrast, infection of BM cells with H2-D^b resulted in no significant rescue of thymocyte development. Therefore, expression of either class I MHC allele confers an advantage on the B cell lineage, whereas expression of H2-K^b but not H2-D^b at levels obtained in

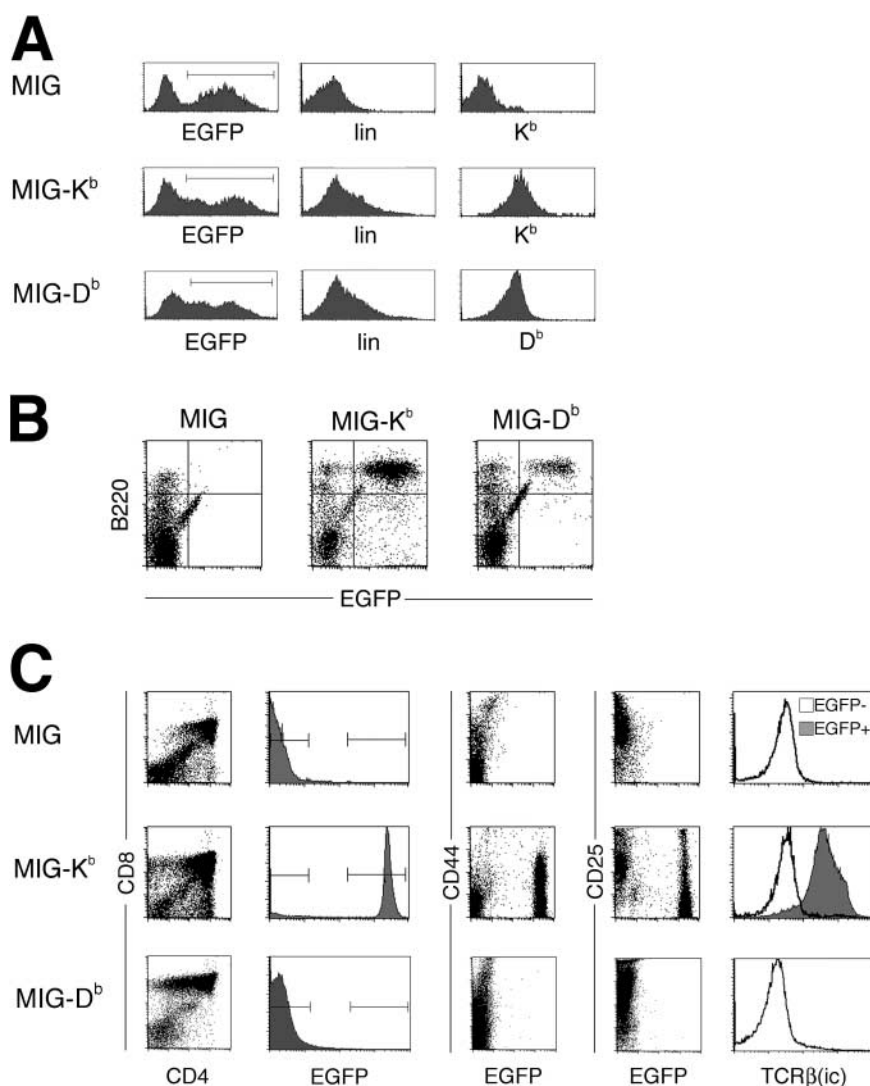


Figure 6. Reconstitution of $K^bD^b^{-/-}$ BM with H2- K^b restores normal thymocyte development. (A) cDNAs for H2- K^b and H2- D^b were generated by RT-PCR and subcloned into MIG-R. BM was harvested from $K^bD^b^{-/-}$ mice and infected in vitro with retrovirus encoding for EGFP and H2- K^b (MIG- K^b), EGFP and H2- D^b (MIG- D^b), or EGFP alone (MIG). Flow cytometry analysis of cultured cells was performed at day 5 of culture after staining with lineage marker antibodies and anti-H2- K^b or anti-H2- D^b antibodies. Data shown in the right two histograms results from gating on EGFP⁺ cells as shown in the left histogram. (B) Equal numbers of EGFP⁺ lin⁻ BM cells infected with the indicated virus in vitro were transferred into sublethally irradiated RAG^{-/-} hosts. Recipient mice were killed at 4 wk, and spleens were analyzed by flow cytometry after staining for B220. (C) Equal numbers of EGFP⁺ lin⁻ BM cells infected with the indicated virus in vitro were transferred into sublethally irradiated RAG^{-/-} hosts. Recipient mice were killed at 4 wk, and thymi were analyzed by flow cytometry after staining for CD4, CD8, CD44, CD25, and intracellular staining for TCR β . Data is representative for two independent experiments with a total of six chimeras for each group.

these experiments confers protection from NK cells on the T cell lineage.

CD8 T Cells Generated in the Presence of NK Cells Remain Susceptible to NK Cell Killing. Although much delayed, CD8 T cells do accumulate in the peripheral lymphatic organs of RAG^{-/-} hosts transferred with OT-I $K^bD^b^{-/-}$ BM and are spared from killing by NK cells. What is the fate of OT-I $K^bD^b^{-/-}$ CD8 T cells that are activated in vivo? LNs from chimeric mice were removed 10 wk after the transfer, and CD8 T cells were enriched by depletion of cells of other lineages by magnetic separation. $K^bD^b^{-/-}$ CD8 T cells display a naive phenotype (CD69 negative, CD44 low/intermediate) and are readily activated in vitro when encountering their cognate antigen (32). For comparison, we examined activation of OT-I $K^bD^b^{+/+}$ CD8 T cells in parallel. CD8 T cells were CFSE-labeled and then transferred into hosts that were subsequently immunized with the activating peptide SIINFEKL. In hosts devoid of NK cells, namely *cyc*^{-/-} or NK cell-depleted B6 hosts, rapid proliferation of both class I MHC-positive and -neg-

ative CD8 T cells was observed 2 d after injection of the antigen, as judged by dilution of CFSE (Fig. 7 A). However, if cells were transferred into hosts not depleted of NK cells, few cells were retrieved from mice transferred with $K^bD^b^{-/-}$ grafts, whereas normal CFSE dilution profiles were observed after transfer of $K^bD^b^{+/+}$ grafts, indicating that the transferred $K^bD^b^{-/-}$ cells were killed by NK cells. Activation of CD8 T cells accelerated their disappearance although complete eradication of grafted CD8 T cells was effectuated even in the absence of an activating stimulus, albeit after a prolonged period of time (not depicted). Quantitation of tetramer-positive CD8 T cells retrieved from the hosts confirmed these results. Although $K^bD^b^{-/-}$ cells expanded to some extent (by 2.7 times) in B6 hosts, expansion lagged far behind that of $K^bD^b^{+/+}$ cells (14.1 times). Almost complete loss of the $K^bD^b^{-/-}$ graft was observed after transfer into RAG^{-/-} hosts. In contrast, in the absence of NK cells, $K^bD^b^{-/-}$ and $K^bD^b^{+/+}$ CD8 T cells expanded to a similar extent (7.3 versus 5.8-fold). We conclude that $K^bD^b^{-/-}$ CD8 T cells in the pe-

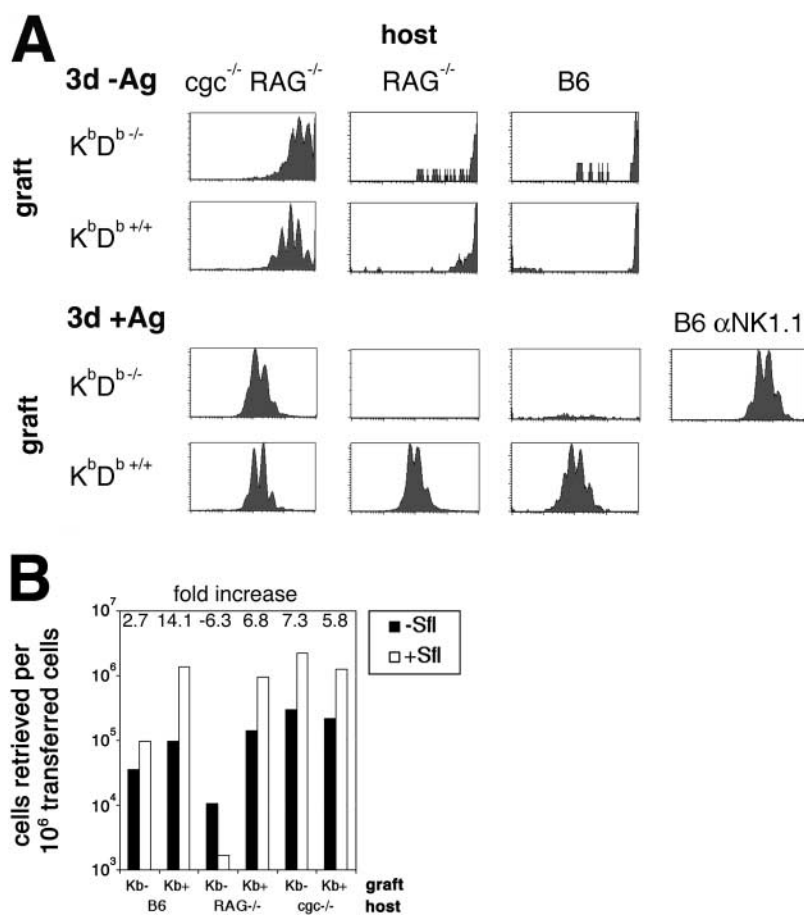


Figure 7. Mature CD8 T cells generated from $K^bD^b^{-/-}$ grafts in the presence of NK cells remain susceptible to NK cell killing. (A) BM from OT-I $K^bD^b^{-/-}$ or OT-I $K^bD^b^{+/+}$ mice was transferred into sublethally irradiated $RAG^{-/-}$ hosts. LN cells were harvested 10 wk after the transfer, enriched for CD8 T cells, labeled with CFSE, and transferred in equal numbers into recipient mice as indicated. Some mice were challenged with the antigenic peptide SIINFEKL i.p. 24 h after the transfer (+Ag). Mice were killed 72 h after the transfer, and spleen and LN (in the case of $RAG^{-/-}$ and B6 mice) were harvested. Cells were stained with anti-CD8 antibody and with K^b (SIINFEKL) tetramer to detect transgenic CD8 T cells. CFSE dilution profiles are shown after gating on $CD8^+$ tetramer⁺ cells. (B) Experimental setup as in A. The relative number of OT-I T cells retrieved from recipient animals is shown for mice grafted with $K^bD^b^{-/-}$ and $K^bD^b^{+/+}$ BM and the effect of challenge with the antigenic peptide (SH) on the number of OT-I cells is indicated above each pair of bars. Data are presented as mean values from duplicate experiments.

riphery remain susceptible to NK cell killing, which is triggered upon activation.

Discussion

The role of NK cells in rejection of class I MHC disparate BM grafts is well established (1, 2). However, little is known about the susceptibility of specific hematopoietic lineages to NK cell killing. Earlier, we showed that mature $K^bD^b^{-/-}$ CD8 T cells develop after transfer of OT-I $K^bD^b^{-/-}$ BM into class I MHC-competent $RAG^{-/-}$ hosts (31, 32). However, the accumulation of these OT-I CD8 T cells in the periphery was much delayed compared with class I MHC-positive grafts. We here studied the kinetics of thymocyte development and production of CD8 T cells in more detail and found a significant delay in generation of DP and SP thymocytes from class I MHC-negative grafts. This delay was caused by NK cell-mediated toxicity. Our results further confirm the existence *in vivo* of a subset of DP thymocytes that is TCR $\alpha\beta$ negative.

DP thymocytes that could be recovered for the first several weeks after BM engraftment corresponded to an unusual intermediate state before such cells move on to the mature DP state displayed in class I MHC-positive grafts. This DP subset was observed not only when using class I MHC-deficient BM from TCR transgenic animals but

also when nontransgenic class I MHC-deficient BM was transferred. These unusual DP thymocytes showed intermediate to high levels of CD25 and lacked detectable TCR β . BrdU labeling showed reduced proliferative activity in the DN and DP compartment of $K^bD^b^{-/-}$ -grafted thymi. We propose that such unusual cells are generated by a pathway much less efficient than the regular, pre-TCR-driven pathway. In all likelihood, the presence of these cells in normal mice would be masked by the overwhelming majority of regular DP thymocytes. However, in a situation in which the majority of cells is depleted selectively, this alternate pathway becomes apparent. The appearance of such cells demonstrates that β selection is not a prerequisite for thymocytes to progress to the DP stage. DN to DP transition in the absence of efficient β selection is found in irradiated RAG -deficient animals (50, 51) and in p53-deficient animals (52, 53) but is consistently accompanied by complete down-regulation of CD25 and by TCR β expression in p53-deficient animals. Although the settings of the model described here are perhaps unusual, the data show that DP cells can also be generated by an alternate pathway that does not involve pre-TCR-mediated β selection. Our data are consistent with the notion that such DP cells can generate mature SP cells. We cannot exclude the formal possibility that expression of minute amounts of TCR β , undetectable by cytofluorimetry in the

DP thymocytes in our model, drives the DN to DP transition. However, the failure to down-regulate CD25 effectively argues against β selection.

The observed phenotype resembles to some extent that of thymocytes from pT α -deficient animals (37). However, in pT α ^{-/-} animals the arrest in development takes place at the DN stage. The absence of $\gamma\delta$ T cells in the thymus of K^bD^b^{-/-}-grafted animals and the failure of a transgenic TCR $\alpha\beta$ receptor to rescue thymocyte development also argue against a role of pT α in “arresting” thymocyte development in the system described here.

Our data show that the majority of thymocytes that lack class I MHC are eliminated by NK cells. Only the slowly dividing cells may escape such killing, presumably because they lack the putative NK-activating ligand present on the rapidly proliferating cells. SP cells generated by this pathway are fully functional, polyclonal, and remain susceptible to NK cell killing *in vivo*. Therefore, clonal expansion of a rare NK cell-resistant precursor cannot account for our results.

The mere absence of class I MHC molecules on the target cells is not sufficient to induce their elimination by NK cells. This finding is in agreement with previously published reports that show that nontransformed cells may be spared from NK cell attack (29). What is the ligand that renders proliferating thymocytes susceptible to NK cell attack? Although it is generally accepted that NK cells require an activating signal in addition to the lack of an inhibitory signal (54), few activating ligands have been described for human- or mouse-activating NK cell receptors. H-60 is now known as an activating ligand for NKG2D (23). An H-60-derived peptide presented by H2-K^b also is responsible for alloreactivity of B6 CD8 T cells against BALB/b targets (22). Binding of H-60 to NKG2D was implicated in targeting of class I MHC-positive activated lymphocytes by NK cells in an *in vitro* model (55). Other MHC-like molecules may exist that display similar functions, and such molecules could evoke NK cell toxicity. Members of the RAE-1 family are other activating ligands for NKG2D (24). Their expression is up-regulated during development and transformation (21, 24, 56), making them likely candidates for up-regulation on proliferating cells. The ligand that renders thymocytes susceptible to NK cell lysis is not known. Thymocytes derived from class I MHC-negative and -positive BM grafts failed to bind an NKG2D-Ig fusion protein (a gift from L. Lanier, University of California, San Francisco, San Francisco, CA; unpublished data). A possible activating ligand therefore likely binds to an activating receptor other than NKG2D.

Alternatively, down-regulation of a ligand for inhibitory receptors might occur. However, all inhibitory ligands for NK receptors described so far are members of the MHC family. We observed the same delay in thymocyte development when using BM from K^bD^b^{-/-}, TAP^{-/-}, and β 2m^{-/-} mice, arguing against a mechanism that involves down-regulation of one of the known ligands for inhibitory NK cell receptors.

Although NK cells can attack BM grafts that do not display the full array of host class I MHC molecules (1), we find that the mere introduction of H2-K^b into K^bD^b^{-/-} BM cells is sufficient to rescue development of thymocytes, whereas introduction of H2-D^b is not. H2-K^b and H2-D^b bind differentially to Ly49 alleles in B6 mice, namely to Ly49A and C (57). It is possible that the effect observed here is unique to the C57/BL6J background, which may be biased toward usage of H2-K^b over H2-D^b as ligands for the NK inhibitory receptors. The possibility remains that H2-D^b expressed at higher levels than attained in our experiments would rescue thymocyte development as well.

What might be the physiological role of selective targeting of certain subpopulations of lymphocytes by NK cells? NK cells regulate the immune response of CD4 T cells during MCMV infection in class I MHC-competent mice (58). Production of effector cytokines, proliferation, and clonal size are down-modulated by NK cells in this model. During LCMV infection, where NK cells do not participate in the antiviral response, such control of CD4 cells is seen in class I MHC-deficient mice. Similar control mechanisms might regulate clonal size and activation of T cell subpopulations during the course of other immune responses or during homeostatic proliferation, even when no or only subtle changes in class I MHC expression are apparent. Activating ligands can override inhibitory signals under certain conditions, as was shown for overexpression of RAE-1 on the class I MHC-positive Ba/F3 cell line (59). Such increased expression of RAE-1 rendered the otherwise resistant Ba/F3 target susceptible to killing by NK cells. Also, NK cells were shown to lyse class I MHC-competent syngeneic T cells activated *in vitro* (55). These data argue for a possible role of NK cells in the regulation of clone size under physiological conditions. These regulatory properties of NK cells need not be limited to their cytolytic activity but might extend to modulation of cytokine release by T cells as well.

In summary, we demonstrate a role of NK cell-mediated toxicity in K^bD^b^{-/-} thymocyte development, with selective survival of an unusual subset of DP thymocytes. Mature CD8 T cells are generated through an alternate pathway that involves less rapid proliferation and delayed TCR β expression. Such CD8 T cells are functional, yet remain susceptible to NK cell killing.

We thank the members of the Ploegh lab for helpful discussions.

This work was supported by a National Institutes of Health grant to H.L. Ploegh (R01 AI33456) and by a fellowship from the Deutsche Forschungsgemeinschaft (Scho 776/1-1) to E. Schott.

Submitted: 5 June 2003

Revised: 29 August 2003

Accepted: 9 September 2003

References

1. Yu, Y.Y., T. George, J.R. Dorfman, J. Roland, V. Kumar, and M. Bennett. 1996. The role of Ly49A and 5E6(Ly49C) molecules in hybrid resistance mediated by murine natural

- killer cells against normal T cell blasts. *Immunity*. 4:67–76.
2. Bix, M., N.S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet. 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature*. 349: 329–331.
 3. Lanier, L.L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359–393.
 4. Yokoyama, W.M. 1995. Natural killer cell receptors. *Curr. Opin. Immunol.* 7:110–120.
 5. Yokoyama, W.M. 1993. Recognition structures on natural killer cells. *Curr. Opin. Immunol.* 5:67–73.
 6. Karre, K., H.G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 319: 675–678.
 7. Ljunggren, H.G., and K. Karre. 1985. Host resistance directed selectively against H-2-deficient lymphoma variants. Analysis of the mechanism. *J. Exp. Med.* 162:1745–1759.
 8. Piontek, G.E., K. Taniguchi, H.G. Ljunggren, A. Gronberg, R. Kiessling, G. Klein, and K. Karre. 1985. YAC-1 MHC class I variants reveal an association between decreased NK sensitivity and increased H-2 expression after interferon treatment or in vivo passage. *J. Immunol.* 135:4281–4288.
 9. Storkus, W.J., D.N. Howell, R.D. Salter, J.R. Dawson, and P. Cresswell. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J. Immunol.* 138: 1657–1659.
 10. Storkus, W.J., J. Alexander, J.A. Payne, J.R. Dawson, and P. Cresswell. 1989. Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes. *Proc. Natl. Acad. Sci. USA*. 86:2361–2364.
 11. Burshtyn, D.N., A.M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J.P. Kinet, and E.O. Long. 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity*. 4:77–85.
 12. Salcedo, M., M. Andersson, S. Lemieux, L. Van Kaer, B.J. Chambers, and H.G. Ljunggren. 1998. Fine tuning of natural killer cell specificity and maintenance of self tolerance in MHC class I-deficient mice. *Eur. J. Immunol.* 28:1315–1321.
 13. Salcedo, M., A.D. Diehl, M.Y. Olsson-Alheim, J. Sundback, L. Van Kaer, K. Karre, and H.G. Ljunggren. 1997. Altered expression of Ly49 inhibitory receptors on natural killer cells from MHC class I-deficient mice. *J. Immunol.* 158:3174–3180.
 14. Olsson-Alheim, M.Y., M. Salcedo, H.G. Ljunggren, K. Karre, and C.L. Sentman. 1997. NK cell receptor calibration: effects of MHC class I induction on killing by Ly49Ahigh and Ly49Alow NK cells. *J. Immunol.* 159:3189–3194.
 15. Hoglund, P., C. Ohlen, E. Carbone, L. Franksson, H.G. Ljunggren, A. Latour, B. Koller, and K. Karre. 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m-mice: nonresponsiveness controlled by beta 2m-bone marrow in chimeric mice. *Proc. Natl. Acad. Sci. USA*. 88: 10332–10336.
 16. Freland, S., B.J. Chambers, M. Andersson, L. Van Kaer, and H.G. Ljunggren. 1998. Rejection of allogeneic and syngeneic but not MHC class I-deficient tumor grafts by MHC class I-deficient mice. *J. Immunol.* 160:572–579.
 17. Cerwenka, A., and L.L. Lanier. 2001. Ligands for natural killer cell receptors: redundancy or specificity. *Immunol. Rev.* 181:158–169.
 18. Aldrich, C.J., A. DeCloux, A.S. Woods, R.J. Cotter, M.J. Soloski, and J. Forman. 1994. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell*. 79:649–658.
 19. Vance, R.E., J.R. Kraft, J.D. Altman, P.E. Jensen, and D.H. Raulet. 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J. Exp. Med.* 188: 1841–1848.
 20. Vance, R.E., A.M. Jamieson, and D.H. Raulet. 1999. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* 190:1801–1812.
 21. Nomura, M., Z. Zou, T. Joh, Y. Takihara, Y. Matsuda, and K. Shimada. 1996. Genomic structures and characterization of Rae1 family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. *J. Biochem. (Tokyo)*. 120:987–995.
 22. Malarkannan, S., P.P. Shih, P.A. Eden, T. Horng, A.R. Zuberi, G. Christianson, D. Roopenian, and N. Shastri. 1998. The molecular and functional characterization of a dominant minor H antigen, H60. *J. Immunol.* 161:3501–3509.
 23. Diefenbach, A., A.M. Jamieson, S.D. Liu, N. Shastri, and D.H. Raulet. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* 1:119–126.
 24. Cerwenka, A., A.B. Bakker, T. McClanahan, J. Wagner, J. Wu, J.H. Phillips, and L.L. Lanier. 2000. Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity*. 12:721–727.
 25. Ono, A., D.B. Amos, and H.S. Koren. 1977. Selective cellular natural killing against human leukaemic T cells and thymus. *Nature*. 266:546–548.
 26. Hansson, M., R. Kiessling, B. Andersson, K. Karre, and J. Roder. 1979. NK cell-sensitive T-cell subpopulation in thymus: inverse correlation to host NK activity. *Nature*. 278: 174–176.
 27. Hansson, M., K. Karre, R. Kiessling, J. Roder, B. Andersson, and P. Hayry. 1979. Natural NK-cell targets in the mouse thymus: characteristics of the sensitive cell population. *J. Immunol.* 123:765–771.
 28. Hoglund, P., R. Glas, C. Menard, A. Kase, M.H. Johansson, L. Franksson, F. Lemmonier, and K. Karre. 1998. Beta2-microglobulin-deficient NK cells show increased sensitivity to MHC class I-mediated inhibition, but self tolerance does not depend upon target cell expression of H-2Kb and Db heavy chains. *Eur. J. Immunol.* 28:370–378.
 29. Sentman, C.L., M.Y. Olsson, M. Salcedo, P. Hoglund, U. Lendahl, and K. Kare. 1994. H-2 allele-specific protection from NK cell lysis in vitro for lymphoblasts but not tumor targets. Protection mediated by alpha 1/alpha 2 domains. *J. Immunol.* 153:5482–5490.
 30. Storkus, W.J., and J.R. Dawson. 1986. B cell sensitivity to natural killing: correlation with target cell stage of differentiation and state of activation. *J. Immunol.* 136:1542–1547.
 31. Schott, E., and H.L. Ploegh. 2002. Mouse MHC class I tetramers that are unable to bind to CD8 reveal the need for CD8 engagement in order to activate naive CD8 T cells. *Eur. J. Immunol.* 32:3425–3434.
 32. Schott, E., N. Bertho, Q. Ge, M.M. Maurice, and H.L. Ploegh. 2002. Class I negative CD8 T cells reveal the confounding role of peptide-transfer onto CD8 T cells stimulated with soluble H2-Kb molecules. *Proc. Natl. Acad. Sci.*

- USA. 99:13735–13740.
33. Shortman, K., and L. Wu. 1996. Early T lymphocyte progenitors. *Annu. Rev. Immunol.* 14:29–47.
 34. von Boehmer, H., I. Aifantis, J. Feinberg, O. Lechner, C. Saint-Ruf, U. Walter, J. Buer, and O. Azogui. 1999. Pleiotropic changes controlled by the pre-T-cell receptor. *Curr. Opin. Immunol.* 11:135–142.
 35. Hoffman, E.S., L. Passoni, T. Crompton, T.M. Leu, D.G. Schatz, A. Koff, M.J. Owen, and A.C. Hayday. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev.* 10:948–962.
 36. Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative T cell selection. *Annu. Rev. Immunol.* 21:139–176.
 37. Fehling, H.J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature.* 375:795–798.
 38. Vugmeyster, Y., R. Glas, B. Perarnau, F.A. Lemonnier, H. Eisen, and H. Ploegh. 1998. Major histocompatibility complex (MHC) class I K^bD^b $-/-$ deficient mice possess functional CD8⁺ T cells and natural killer cells. *Proc. Natl. Acad. Sci. USA.* 95:12492–12497.
 39. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell.* 76:17–27.
 40. Garboczi, D.N., U. Utz, P. Ghosh, A. Seth, J. Kim, E.A.E. VanTienhoven, W.E. Biddison, and D.C. Wiley. 1996. Assembly, specific binding, and crystallization of a human TCR- α/β with an antigenic Tax-peptide from human T lymphotropic virus type 1 and the class I MHC molecule HLA-A2. *J. Immunol.* 157:5403–5410.
 41. Tough, D.F., and J. Sprent. 1994. Turnover of naive- and memory-phenotype T cells. *J. Exp. Med.* 179:1127–1135.
 42. Cherry, S.R., D. Biniszkiwicz, L. van Parijs, D. Baltimore, and R. Jaenisch. 2000. Retroviral expression in embryonic stem cells and hematopoietic stem cells. *Mol. Cell. Biol.* 20:7419–7426.
 43. Ory, D.S., B.A. Neugeboren, and R.C. Mulligan. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proc. Natl. Acad. Sci. USA.* 93:11400–11406.
 44. Screpanti, V., R.P. Wallin, H.G. Ljunggren, and A. Grandien. 2001. A central role for death receptor-mediated apoptosis in the rejection of tumors by NK cells. *J. Immunol.* 167:2068–2073.
 45. Taylor, M.A., B. Ward, J.D. Schatzle, and M. Bennett. 2002. Perforin- and Fas-dependent mechanisms of natural killer cell-mediated rejection of incompatible bone marrow cell grafts. *Eur. J. Immunol.* 32:793–799.
 46. Zamai, L., M. Ahmad, I.M. Bennett, L. Azzoni, E.S. Alnemri, and B. Perussia. 1998. Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells. *J. Exp. Med.* 188:2375–2380.
 47. Buer, J., I. Aifantis, J.P. DiSanto, H.J. Fehling, and H. von Boehmer. 1997. Role of different T cell receptors in the development of pre-T cells. *J. Exp. Med.* 185:1541–1547.
 48. Lacorazza, H.D., C. Tucek-Szabo, L.V. Vasovic, K. Remus, and J. Nikolich-Zugich. 2001. Premature TCR alpha beta expression and signaling in early thymocytes impair thymocyte expansion and partially block their development. *J. Immunol.* 166:3184–3193.
 49. Lawlor, D.A., J. Zemmour, P.D. Ennis, and P. Parham. 1990. Evolution of class-I MHC genes and proteins: from natural selection to thymic selection. *Annu. Rev. Immunol.* 8:23–63.
 50. Guidos, C.J., C.J. Williams, G.E. Wu, C.J. Paige, and J.S. Danska. 1995. Development of CD4⁺CD8⁺ thymocytes in RAG-deficient mice through a T cell receptor beta chain-independent pathway. *J. Exp. Med.* 181:1187–1195.
 51. Zuniga-Pflucker, J.C., D. Jiang, P.L. Schwartzberg, and M.J. Lenardo. 1994. Sublethal gamma-radiation induces differentiation of CD4⁻/CD8⁻ into CD4⁺/CD8⁺ thymocytes without T cell receptor beta rearrangement in recombinase activation gene 2^{-/-} mice. *J. Exp. Med.* 180:1517–1521.
 52. Jiang, D., M.J. Lenardo, and C. Zuniga-Pflucker. 1996. p53 prevents maturation to the CD4⁺CD8⁺ stage of thymocyte differentiation in the absence of T cell receptor rearrangement. *J. Exp. Med.* 183:1923–1928.
 53. Haks, M.C., P. Krimpenfort, J.H. van den Brakel, and A.M. Kruisbeek. 1999. Pre-TCR signaling and inactivation of p53 induces crucial cell survival pathways in pre-T cells. *Immunity.* 11:91–101.
 54. Lanier, L.L. 2001. On guard—activating NK cell receptors. *Nat. Immunol.* 2:23–27.
 55. Rabinovich, B.A., J. Li, J. Shannon, R. Hurren, J. Chalupny, D. Cosman, and R.G. Miller. 2003. Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. *J. Immunol.* 170:3572–3576.
 56. Masuda, H., Y. Saeki, M. Nomura, K. Shida, M. Matsumoto, M. Ui, L.L. Lanier, and T. Seya. 2002. High levels of RAE-1 isoforms on mouse tumor cell lines assessed by anti-“pan” RAE-1 antibody confer tumor susceptibility to NK cells. *Biochem. Biophys. Res. Commun.* 290:140–145.
 57. Michaelsson, J., A. Achour, M. Salcedo, A. Kase-Sjostrom, J. Sundback, R.A. Harris, and K. Karre. 2000. Visualization of inhibitory Ly49 receptor specificity with soluble major histocompatibility complex class I tetramers. *Eur. J. Immunol.* 30:300–307.
 58. Su, H.C., K.B. Nguyen, T.P. Salazar-Mather, M.C. Ruzek, M.Y. Dalod, and C.A. Biron. 2001. NK cell functions restrain T cell responses during viral infections. *Eur. J. Immunol.* 31:3048–3055.
 59. Cerwenka, A., J.L. Baron, and L.L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. USA.* 98:11521–11526.