Elimination In Vivo of Developing T Cells by Natural Killer Cells

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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-Kb 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 RA–E1 (21) and H–60 (22), molecules with
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 KDRm−/− 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 TCRβ chain occurs, and a pre–TCR complex consisting of TCRβ and the invariant pTα 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 TCRαβ chain occurs. The resulting TCRαβ complex is the entity that mediates positive and negative selection (for reviewed see reference 36). In the absence of pTα, DN thymocytes are arrested at the CD44+CD25+ stage (DN3) and lack TCRβ expression (37). Few cells overcome this block, and rather large numbers of γδ 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 (H2b), C57/BL6J RAG−/−, C57/BL6J TAP−/−, and C57/BL6J β2m−/− mice were purchased from Jackson Laboratories. RAG−/− common gamma chain (cγc)−/− mice and C57/BL6J perforin−/− mice were obtained from Taconic. KDRm−/− mice have been described (38); they were backcrossed 10 times to C57/BL6J littermates and then intercrossed to obtain KDRm−/− animals. OT-I KDRm−/− mice were generated by breeding OT-I mice (39) to KDRm−/− 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 Vα2, Vβ5, CD8, B220, H2-Kb, and H2-Dα (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 10⁶ cells were transferred into sublethally irradiated (600 rad) RAG−/− mice. Recipients were fed thymeporin/sulmethoxaxol 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 KDRm−/− 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−/− mice. Recipients 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 by 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-TCRβ, PE–anti-Vα2, FITC–anti-Vβ5, PE–anti-CD4, CyC–anti-CD8, CyC–anti-CD44, FITC–anti-CD25, CyC–anti-B220, PE–anti-Kb, PE–anti-Dα, 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 CD3ε, 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 Vβ chains was performed with the mouse Vβ TCR screening panel from Becton Dickinson. For intracellular staining, cells were first stained for surface markers, followed by fixation in 0.5% paraffomaldehyde and permeabilization in 0.5% saponin (Sigma-Aldrich). Staining was then performed with CyC–anti-TCRβ or APC–anti-IFNγ (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.

Kb-SIINFEKL tetramers were produced as described (40). In brief, recombinant H2-Kb and β2m 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) and DNase treatment (50 Kunitz U/ml) as described (41). BrdU incorporation was detected by staining with FITC-anti-BrdU (1 µg/10^6 cells; eBioscience), in the presence of 0.5% Tween20, followed by flow cytometry.

Retroviral Infection of BM Cells. The H2-Kb and H2-Dd cDNAs were amplified by RT-PCR from mRNA isolated from B6 splenocytes with Superscript II reverse transcriptase and Pfu polymerase (both from Invitrogen) using the appropriate primers to introduce a 5' Xhol (Kb) or EcoRI (Dd) and a 3' EcoRI site into the PCR product. The fragments were subcloned into pSP72 (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-Kb, and MIG-Dd.

Virus was propagated by transfecting the constructs MIG, MIG-Kb, and MIG-Dd 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 µg/ml β-mercaptoethanol, penicillin/streptomycin, 25 ng/ml IL-6, 25 ng/ml IL-7, 50 ng/ml CSF, and 50 µg/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 µg/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. KdDb+/− OT-I CD8 T cells were generated as described (32). In brief, OT-I KdDb+/− BM was transferred into sublethally (600 rad) irradiated RAG−/− hosts. LNs were harvested 10 wk later. KdDb+/− or KdDb+/+ 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 µM, 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 µg SINFEKL 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-Kb(SIINFEKL)-tetramer and CyC-anti-sca-1 to establish the frequency of progenitor cells. (B) BM from KdDb+/− or KdDb+/+ 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 TCRβ.

(C) The total number of thymocytes retrieved at different time points after transfer of KdDb+/− or KdDb+/+ BM is indicated. (D) Spleen cells from chimeras that had received KdDb+/− or KdDb+/+ BM were analyzed by flow cytometry 10 wk after BM transfer. (E) BM from KdDb+/−, β2m−/−, TAP−/−, or KdDd+/+ (B6) mice was transferred into sublethally irradiated RAG−/− hosts. Thymi were analyzed at 4 wk by flow cytometry after staining for CD4 and CD8.

Figure 1. Generation of DP and SP thymocytes from KdDb+/− BM grafts is delayed. (A) BM from KdDb+/− and KdDb+/+ mice was stained with lineage marker antibodies (lin) and with antibodies against c-kit and
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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^D^{b/-} or K^D^{b/+} C57/BL6/J (H-2^b) mice were transferred into sublethally irradiated C57/BL6/J 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^D^{b/-} stem cells showed a pronounced delay in development of SP thymocytes compared with mice that received K^D^{b/+} stem cells (Fig. 1 B). No SP thymocytes were detected until week 5, whereas such cells appeared in thymi of K^D^{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^D^{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^D^{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 B2m^-/- mice (Fig. 1 E). Although in TAP^-/- and B2m^-/- 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^D^{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^D^{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^D^{b/-} BM, staining of infiltrating NK cells for intracellular IFNγ content showed little IFNγ 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^D^{b/-} and K^D^{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^D^{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 TCRβ expression in the early course of thymic development, as judged from intracellular and surface staining for TCRβ (Fig. 3 A). Although they up-regulated TCRβ 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 TCRβ rearrangement and TCRβ 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 pTα–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-TCRα (pTα) (37). We hypothesized that early expression of a transgenic TCR should rescue thymic development if insufficient pTα expression and β selection were responsible for the observed developmental delay (47). To that end, OT-I transgenic K^D^{b/-} mice were analyzed. The transfer of OT-I K^D^{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
were readily detected in the vast majority of thymocytes derived from OT-I KbDb\(^+/-\) stem cells as early as 2 wk after transfer, no such expression was found in the thymocytes derived from their OT-I KbDb\(^+/-\) counterparts. Also, OT-I KbDb\(^+/-\) thymocytes showed the same defect in down-regulating the expression of CD25 (not depicted) as seen in the nontransgenic KbDb\(^+/-\) grafts. Although the potency of a TCRp\(\alpha\) 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 TCRp\(\alpha\) argues against a role for pT\(\alpha\) as the underlying mechanism. Also, no increased numbers of TCRp\(\gamma\)-expressing thymocytes were observed (not depicted).

Figure 2. The delay in development of KbDb\(^+/-\) thymocytes is due to NK cell toxicity. (A) BM from KbDb\(^+/-\) 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 TCRp\(\beta\). (B) BM from OT-I KbDb\(^+/-\) or OT-I KbDb\(^+/-\) 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 Kb(SIINFEKL) tetramer. (C) BM from KbDb\(^+/-\) or KbDb\(^+/-\) 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\(\gamma\). (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.
We analyzed the normal course of expression of class I MHC in developing thymocytes. Although high levels of H2-K^k were detected in DN and SP thymocyte populations of K^b/D^b/+/+ grafted thymi, almost complete down-regulation of H2-K^k was found in DP thymocytes, resembling staining patterns of K^b/D^b/-/- thymocytes (Fig. 3 C). The H2-K^k-positive population seen in DN thymocytes from K^b/D^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-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^b/D^b/-/- grafts result from a pathway of maturation that involves less proliferative activity than is the case for regular K^b/D^b/+/+ DP thymocytes.

K^b/D^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^b/D^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^b/D^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^b/D^b/-/- grafts to down-regulate CD25 and express TCRB, suggests that the DP thymocytes generated from a K^b/D^b/-/- graft in the presence of NK cells develop along an alternative pathway that does not involve rapid proliferation and efficient TCRB 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^b/D^b/-/- BM are derived from a single clone of progenitor cells that has acquired resistance to NK cell toxicity, we analyzed the expression of VB chains in peripheral T cells. To that end, cells were harvested from the spleen 8 wk after BM transfer.
The complete absence of NK cells in cKbDb 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, and stained with a panel of Vβ antibodies. Although slight differences in the frequency of expression were noted for the Vβ chains tested, multiple Vβ chains were detected and clearly demonstrate the polyclonal origin of CD4 and CD8 T cells generated from KβDb−/− 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 KβDb−/− BM into RAG−/− cyc−/− animals 3 wk after the initial BM transfer. The complete absence of NK cells in cyc−/− mice ensured that newly generated T cells were not eradicated by NK cells.

Figure 5. Phenotypically distinct KbDb−/− thymocytes generated in the presence on NK cells give rise to polyclonal mature T cells. (A) BM from KβDb−/− or KβDb−/+ mice was transplanted 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-Vβ antibodies. The percentage of spleen cells staining with each individual anti-Vβ antibody is shown for animals grafted with KβDb−/− or KβDb−/+ BM. (B) BM from KβDb−/− or KβDb−/+ mice was transplanted 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−/− cyc−/− 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-Vα2, and anti-Vβ5 antibodies.

Analysis of the spleens of the different chimeras showed that BM infected with MIG-Kβ or MIG-Dβ gave rise to EGFP-expressing B cells, whereas no such B cells were observed in animals that had received MIG-infected class I MHC−/− BM (Fig. 6 B), establishing that chimeras were successfully generated from BM infected with MIG-Kβ and MIG-Dβ. 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-Kβ 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-Kβ—positive cells matured normally in the thymus, down-regulated CD25, and up-regulated TCRβ, whereas nontransduced (EGFP−) thymocytes displayed the immature phenotype described above. In contrast, infection of BM cells with H2-Dβ 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-Kβ but not H2-Dβ at levels obtained in...
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\(^{b}\)D\(^{b}\)−/− BM and are spared from killing by NK cells. What is the fate of OT-I K\(^{b}\)D\(^{b}\)−/− 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\(^{b}\)D\(^{b}\)−/− 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\(^{b}\)D\(^{b}\)+/+ 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 c\(^{-}\)/c\(^{-}\) or NK cell-depleted B6 hosts, rapid proliferation of both class I MHC–positive and –negative 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\(^{b}\)D\(^{b}\)−/− grafts, whereas normal CFSE dilution profiles were observed after transfer of K\(^{b}\)D\(^{b}\)+/+ grafts, indicating that the transferred K\(^{b}\)D\(^{b}\)−/− 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\(^{b}\)D\(^{b}\)−/− cells expanded to some extent (by 2.7 times) in B6 hosts, expansion lagged far behind that of K\(^{b}\)D\(^{b}\)+/+ cells (14.1 times). Almost complete loss of the K\(^{b}\)D\(^{b}\)−/− graft was observed after transfer into RAG−/− hosts. In contrast, in the absence of NK cells, K\(^{b}\)D\(^{b}\)−/− and K\(^{b}\)D\(^{b}\)+/+ CD8 T cells expanded to a similar extent (7.3 versus 5.8-fold). We conclude that K\(^{b}\)D\(^{b}\)−/− CD8 T cells in the pe-
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 KbDb+/H11002/+/H11002/+/H11002 CD8 T cells develop after transfer of OT-I KbDb+/H11002/+/H11002 BM into class I MHC–competent RAG+/H11002/+/H11002 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 TCRαβ 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 TCRαβ. BrdU labeling showed reduced proliferative activity in the DN and DP compartment of KbDb+/H11002/+/H11002–grafted thymi. We propose that such unusual cells are generated by a pathway much less efficient than the regular, pre-TCR–mediated 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 TCRαβ selection is not a prerequisite for thymocytes to progress to the DP stage. DN to DP transition in the absence of efficient TCRαβ 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 TCRβ 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 TCRαβ 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 TCRβ, undetectable by cytofluorimetry in the

Figure 7. Mature CD8 T cells generated from KbDb+/− grafts in the presence of NK cell killing. (A) BM from OT-I KbDb+/− or OT-I KbDb+/+ 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 SHINFEKL 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 Kb(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 KbDb+/− and KbDb+/+ 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.
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 pTα-deficient animals (37). However, in pTα−/− animals the arrest in development takes place at the DN stage. The absence of γδ T cells in the thymus of K\(^{a}D\beta^{−/−}\)-grafted animals and the failure of a transgenic TCRαβ receptor to rescue thymocyte development also argue against a role of pTα 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 NK 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.

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\(^{a}D\beta^{−/−}\)-grafted animals 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\(^{a}D\beta^{−/−}\) 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 TCRβ expression. Such CD8 T cells are functional, yet remain susceptible to NK cell killing.

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