Hematopoietic Cells and Radioresistant Host Elements
Influence Natural Killer Cell Differentiation

By M. Sykes,* M. W. Harty,* F. M. Karlhofer,† D. A. Pearson,*
Gregory Szot,* and W. Yokoyama‡

From the *Transplantation Research Biology Center, Surgical Service, Massachusetts General
Hospital, Harvard Medical School, Boston, Massachusetts 02129; and the ‡Department of
Medicine and Brookdale Center for Molecular Biology, Mt. Sinai Medical Center,
New York 10029-6574

Summary
Radioresistant host elements mediate positive selection of developing thymocytes, whereas bone
marrow-derived cells induce clonal deletion of T cells with receptors that are strongly autoreactive.
In contrast to T cell development, little is known about the elements governing the natural
killer (NK) cell repertoire, which, similar to the T cell repertoire, differs between individuals
bearing different major histocompatibility complex (MHC) phenotypes. We have used murine
bone marrow transplantation models to analyze the influence of donor and host MHC on an
NK cell subset. We examined the expression of Ly-49, which is strongly expressed on a subpopulation
of NK cells of H-2b mice, but not by NK cells of H-2a mice, probably because of a negative
effect induced by the interaction of Ly-49 with Dα. To evaluate the effect of hematopoietic cell
H-2a expression on Ly-49 expression of H-2b NK cells, we prepared mixed allogeneic chimeras
by administering T cell-depleted allogeneic (B10.A, H-2a) and host-type (B10, H-2b) marrow
to lethally irradiated B10 mice, or by administering B10.A marrow to B10 recipients conditioned
by a nonmyeloablative regimen. Expression of H-2a on bone marrow–derived cells was sufficient
to downregulate Ly-49 expression on both H-2a and H-2b NK cells. This downregulation was
thymus independent. To examine the effect of H-2a expression on only radioactive host
elements, we prepared fully allogeneic chimeras by administering B10 bone marrow to lethally
irradiated B10.A recipients. B10 NK cells of these fully allogeneic chimeras also showed
downregulation of Ly-49 expression. The lower level of H-2a expressed on H-2a × H-2b F1 cells
induced more marked downregulation of Ly-49 expression on B10 NK cells when presented
donor marrow in mixed chimeras than when expressed only on radioresistant host cells. Our
studies show that differentiation of NK cells is determined by interactions with MHC molecules
expressed on bone marrow–derived cells and, to a lesser extent, by MHC antigens expressed
on radioresistant host elements.

T cells undergo negative and positive selection during de-
development, resulting in self-MHC-restricted recognition
of peptide antigens, and deletion of T cell clones with strong
reactivity to self-antigens. Positive selection is thought to be
mediated predominantly by thymic epithelial cells, whereas
clonal deletion of self-reactive T cell progenitors is mediated
primarily by bone marrow–derived cells populating the thymus
(1, 2). Less is known about the selection processes which NK
cells undergo during their differentiation. Although patterns
of NK cell–mediated recognition are known to differ between
MHC-disparate mouse strains (3) and human individuals (4),
the environmental determinants of the NK cell repertoire have
yet to be defined. We have used radiation bone marrow
chimeras to address separately the influence of bone marrow–
derived cells and of radioresistant host elements on NK cell
expression of a surface molecule that recognizes MHC class
I antigens and regulates cytolytic activity.

Ly-49 is a recently defined surface molecule expressed on
a subpopulation of NK cells (5). The putative interaction of
Ly-49 with D4 or D6 class I MHC molecules on target cells
globally inhibits cytosis by Ly-49+ NK cells suggesting
that Ly-49 is an inhibitory NK cell receptor specific for these
MHC class I antigens (5). Presumably resulting from a negative
effect induced by recognition of D4 or D6, Ly-49 is not de-
tected on NK cells of animals bearing these MHC antigens
(6). We took advantage of this observation to evaluate the
effect of D4 expression on bone marrow or radioresistant
host elements on NK cell Ly-49 expression.
Materials and Methods

**Animals.** C57BL/10 (B10, H-2 b, Kk b/D d), B10.A (H-2 b, Kk b/D d), A/JScNCR (A/J, H-2 b) and (B10 x B10.A)F1 (H-2 b x H-2 b) mice were purchased from Frederick Cancer Research Facility, (Frederick, MD). B10.BR (H-2 b, Kk b/D d) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in sterile microisolator cages in a viral antibody-free environment.

**Bone Marrow Transplantation (BMT).** Chimeras were prepared as described (7) by reconstituting 10.25 Gy whole body irradiated (WBI) (1.03 Gy/min, 137Cs source) B10, B10.A, or (B10 x B10.A)F1, mice, or 9.5 Gy WBI A/J mice with the indicated T cell-depleted (TCD) marrow inocula. T cell depletion of bone marrow cells (BMC) was carried out as described (8). All bone marrow inocula were T cell depleted, unless otherwise indicated.

Another BMT model involved administration of 5 x 10^5 non-TCD BMC to B10.BR mice conditioned with sublethal (7 Gy) total body irradiation (TBI) and pretreated with deleterious doses of anti-NK.1 mAb PK136 on days −6 and −1 before BMT. These animals showed complete donor-type reconstitution in the lymphoid and myeloid lineages.

A third BMT model involved administration of 15 x 10^5 non-TCD B10.A or A/J BMC to B10 mice pretreated with anti-CD4 and anti-CD8 Abs, 7 Gy thymic irradiation, and 3 Gy TBI. This nonmyeloablative conditioning regimen led to induction of permanent mixed chimerism after BMT, as previously described (10).

**Thymectomy.** Animals were anesthetized intraperitoneally with pentobarbital and thymectomized by en bloc excision after a partial median sternotomy, as described (11).

**Abs and Flow Cytometry (FCM).** FCM analyses were performed on a FACScan® or FACSort® (Becton Dickinson & Co., Mountain View, CA). Chimerism was evaluated by FCM analysis of peripheral white blood cells (WBC) prepared by hypotonic lysis of RBC. WBC were stained with FITC- or biotin-conjugated (plus PE-streptavidin [PEA]) D e-specific mouse IgG2a mAb 34-2-12 (12), K k-specific mouse IgG2a mAb 16-1-11 (13), or K k-specific mouse IgG2a mAb 36-7-5 (13) to distinguish H-2 k (D e-positive, K k-negative) cells. Nonreactive IgG2a mAb HOPC1 was used as a negative control. Chimerism was evaluated for lymphocytes, monocytes, and granulocytes, which could be distinguished on the basis of forward angle versus 90° light scatter characteristics.

For analysis of Ly-49 expression on NK cells, PBL were prepared and labeled with anti-Ly-49 mAb A1-FITC (14) and NK.1-specific IgG2a mAb PK136-biotin (PharMingen, San Diego, CA) plus PEA, or with PK136-PE (PharMingen). To determine the percentage of NK.1.1 + cells that were Ly-49 +, 10,000 gated PK136 + cells were collected. The percentage of Ly-49 + NK cells that expressed TCR-α/β, 10,000 gated PK136 + cells were collected. The percentage of Ly-49 + NK cells that expressed H-2 k (D e-positive, K k-negative) cells. Nonreactive IgG2a mAb HOPC1 was used as a negative control.

Chimerism was evaluated for lymphocytes, monocytes, and granulocytes, which could be distinguished on the basis of forward angle versus 90° light scatter characteristics.

For analysis of Ly-49 expression on NK cells, PBL were prepared and labeled with anti-Ly-49 mAb A1-FITC (14) and NK.1-specific IgG2a mAb PK136-biotin (PharMingen, San Diego, CA) plus PEA, or with PK136-PE (PharMingen). To determine the percentage of NK.1.1 + cells that were Ly-49 +, 10,000 gated PK136 + cells (distinguished by staining above negative control mAb HOPC1-biotin/PEA or Leu4-PE) were collected for analysis of staining with A1-FITC. The percentage of gated PK136 + cells staining with control antibody HOPC1-FITC was subtracted from the percentage of gated PK136 + cells staining with A1-FITC. PBL were also stained with negative control mouse IgG2a mAb HOPC1-FITC versus PK136-biotin/PEA and 36-7-5 (anti-K k)-FITC versus PK136-biotin/PEA (or PK136-PE).

For three-color FCM analysis, PBL were stained with combinations of a FITC-, a PE-, and a biotin-conjugated mAb with CyChrome-streptavidin (Cy-SA; PharMingen). FITC-conjugated mAbs were HOPC-1 (control) and A1; PE-conjugated mAbs were anti-human CD3 mAb Leu4 (negative control; Becton Dickinson & Co.) and PK136; biotin-conjugated antibodies were hamster IgG (Ham IgG), anti-TCR-α/β mAb H57-597 (15) (PharMingen), HOPC-1 (control), and anti-D e mAb 34-2-12. To determine the percentage of Ly-49 + NK cells that expressed TCR-α/β, 10,000 gated PK136 + cells were collected. The percentage of Ly-49 + PK136 + cells staining with Ham IgG-Cy-SA was subtracted from the percentage of Ly-49 + PK136 + cells staining with H57-597-Cy-SA. To analyze Ly-49 expression on B10 NK cells in PBL of chimeras, 10,000 gated NK.1.1 + cells were collected, and D e-, NK.1.1 + cells were selected on FL2 x FL3 plots for comparison of green fluorescence staining with A1-FITC to background staining with HOPC1-FITC.

**Results and Discussion**

**Recovery of Ly-49 + NK Cells in Syngeneic (B10→B10) BMT Recipients.** We used two-color FCM with Ly-49-specific mAb A1 to evaluate Ly-49 expression on NK.1.1 + cells of chimeras prepared in the B10.A (H-2 b, D e)/B10 (H-2 b, D b) strain combination. At all time points tested 7–30 wk after BMT, B10→B10 BMT recipients showed similar percentages of Ly-49 + NK cells to those in B10 controls tested in the same assay (Figs. 1 f and 2). Although "dull" Ly-49 staining was variably detected on NK cells of D e-bearing H-2 b B10.A, H-2 b × H-2 b (B10 × B10.A)F1, or H-2 b B10.BR mice (e.g., Ly-49 staining producing a dull "shoulder" above background staining on B10.A NK cells had a peak fluorescence of 10 channels, compared with 41 channels for the distinct peak produced by Ly-49 staining of B10 NK cells in the same assay), brightly staining Ly-49 + cells were not detected on normal mice of these strains, consistent with previous results (6). The reason for this variable dull Ly-49 expression is not known, and is currently under investigation.

A subpopulation of NK.1.1 + cells expresses TCR-α/β (16–18). We used three-color FCM analysis to determine the percentage of NK.1.1 +, Ly-49 + cells that expressed TCR-α/β in PBL of three B10→B10 BMT recipients and a normal B10 mouse. Only 1.22–6.01% of NK.1.1 + Ly-49 + cells had a peak fluorescence of 10 channels, compared with 41 channels for the distinct peak produced by Ly-49 staining of B10 NK cells in the same assay), brightly staining Ly-49 + cells were not detected on normal mice of these strains, consistent with previous results (6). The reason for this variable dull Ly-49 expression is not known, and is currently under investigation.

**H-2 Bone Marrow Downregulates Ly-49 Expression on H-2 b NK Cells.** To examine the effect of hematopoietic cell H-2 b (D b) expression on Ly-49 expression by D e- NK cells, we prepared mixed chimeras by reconstituting lethally irradiated B10 mice with a mixture of TCD allogeneic and syngeneic (host type) marrow obtained from B10 and B10.A donors (i.e., B10+B10.A→B10). These mixed chimeras show specific T cell tolerance to antigens of the donor and of the host (19). The percentage of NK.1.1 + cells in PBL of control and chimeric animals was similar (3.4 ± 1.3% SD for normals; 2.8 ± 1% SD for mixed chimeras; P = 0.1), and two-color FCM analysis showed that the proportion of NK cells of donor and host type was similar to the proportion of total PBL of each type. For example, in a chimera in which 48.5% of PBL
were Dd positive (i.e., of B10.A origin), 44.2% of NK1.1+ cells were Dd positive; in a chimera in which 80% of PBL were Dd positive, 81.2% of NK1.1+ cells were Dd positive.

NK cells from B10+B10.A→B10 chimeras analyzed 7-29 wk after BMT did not express Ly-49 above levels seen in B10.A controls. Similar results were observed in B10+B10.A→B10.A chimeras (Fig. 1, g and h, respectively, and Fig. 2). Fig. 1, c and d show the presence of B10.A (Kk+) and of B10 (Kk-) NK cells in PBL of both animals presented in Fig. 1, g and h.

Heterozygous expression of H-2~ on (B10 x B10.A)F1 donors also markedly reduced Ly-49 expression on B10 NK cells in mixed chimeras prepared in B10 recipients (Figs. 2 and 3). Similar results were obtained in spleens and PBL from lethally irradiated B10 mice reconstituted with TCD A/J (H-2L Dd+) plus TCD B10 BMC analyzed 15-29 wk after BMT (data not shown). The marked reduction in Ly-49 expression on NK cells of mixed chimeras prepared in B10 recipients shows that the presence of H-2~ bone marrow–derived cells is sufficient to downregulate Ly-49 expression on both H-2b and H-2~ NK cells.

In another model, tolerant mixed allogeneic chimeras prepared by administering B10.A or A/J BMC to B10 recipients conditioned by a nonmyeloablative regimen involving 3 Gy...
TBI, 7 Gy thymic irradiation, and administration of anti-CD4 plus anti-CD8 mAbs as previously described (10), showed a similar downregulation of Ly-49 expression on B10 NK cells 3 and 20 wk after BMT (data not shown). Therefore, engraftment of H-2k BMC without lethal irradiation was sufficient to downregulate Ly-49 expression on B10 NK cells as early as 3 wk after BMT.

Similiar downregulation of Ly-49 expression was also observed in chimeras prepared by this regimen after adult thymectomy (data not shown), indicating that bone marrow–derived cells can exert their downregulatory influence on Ly-49 expression at an extrathymic site.

A previous study showed that administration of β2 microglobulin-negative, class I MHC–deficient bone marrow to class I–expressing recipients results in a pattern of NK cell function that resembles that of the class I–deficient donors (20). Our Ly-49 studies in mixed chimeras demonstrate for the first time that MHC molecules expressed on bone marrow–derived cells can influence the differentiation of NK cells not expressing these MHC molecules. This result suggests that expression of alloantigen on donor bone marrow–derived cells might tolerize host-type NK cells that could otherwise be alloreactive against donor marrow. Since mixed chimerism confers several advantages over completely allogeneic reconstitution as an approach to tolerance induction across both allogeneic and xenogeneic barriers (19, 21), the possible ability of donor marrow–derived cells to tolerize host-derived NK cells could have important implications, particularly in xenotransplantation, in which NK cells present an even greater barrier than they do for allogeneic BMT (10, 21).

Expression of H-2d on Radioresistant Host Elements Downregulates Ly-49 Expression on B10 NK Cells. To determine whether Dd expression on radioresistant host elements could also downregulate Ly-49 expression, we studied fully allogeneic TCD B10→B10.A (10.25 Gy WBI) chimeras, in which Dd is expressed only by the host. In these chimeras, Ly-49 expression was markedly downregulated on B10 NK cells evaluated 4–39 wk after BMT (Figs. 4f and 2).

A small proportion of host-type cells is detectable in the PBL of allogeneic chimeras of this type (22) (Fig. 4d). To rule out the possibility that these residual host-type cells are required for Ly-49 downregulation, we performed similar studies in B10.A recipients of non-TCD B10 BMC in which no host-type lymphohematopoietic cells are detectable (22) (compare Fig. 4g to syngeneic B10→B10 control, Fig. 4a). These animals also showed marked downregulation of Ly-49 expression on B10 NK cells in the first time that MHC molecules expressed on bone marrow–derived cells can influence the differentiation of NK cells not expressing these MHC molecules. This result suggests that expression of alloantigen on donor bone marrow–derived cells might tolerize host-type NK cells that could otherwise be alloreactive against donor marrow. Since mixed chimerism confers several advantages over completely allogeneic reconstitution as an approach to tolerance induction across both allogeneic and xenogeneic barriers (19, 21), the possible ability of donor marrow–derived cells to tolerize host-derived NK cells could have important implications, particularly in xenotransplantation, in which NK cells present an even greater barrier than they do for allogeneic BMT (10, 21).

Figure 4. Expression of H-2d on radioresistant host elements leads to downregulated Ly-49 expression on NK1.1+ cells. (a, d, and g) Kk expression on PBL of B10(-T) (i.e., TCD B10→B10, B10(-T)→B10.A, and B10(+T) (i.e., non-TCD B10→B10.A BMT recipients, respectively. A small population of surviving Kk+B10.A cells can be detected in PBL from B10.A recipients of TCD B10, but not of non-TCD B10 marrow (d vs. g). (b, e, and h) Control staining with FITC-HOPC1 mAb for gated NK1.1+ cells of these BMT recipients. (c, f, and i) Staining of the same gated NK1.1+ populations with anti-Ly-49 mAb A1-HTC.

Environmental Influences on Natural Killer Cell Differentiation
NK cells, we performed similar analyses in lethally irradiated (B10 x B10.A)F1 recipients of TCD B10 BMC. Although the percentage of Ly-49+ NK cells and the intensity of Ly-49 staining in these mice was significantly reduced compared with syngeneic controls (percentages summarized in Fig. 2; typical FCM histograms shown in Fig. 3), this reduction was much less marked than that observed for B10 NK cells developing in homozygous B10.A recipients, or for B10 NK cells in B10+(B10 x B10.A)F1→B10 BMC recipients (Figs. 2 and 3). These results suggest that a higher level of H-2 expression is required on the radiosensitive host environment than on bone marrow–derived cells in order to produce a similar degree of Ly-49 downregulation on H-2 NK cells.

Thus, the MHC phenotype of the host environment influences NK cell Ly-49 expression, but to a lesser degree than does the MHC of engrafted bone marrow. Preliminary results of functional studies using the RMA tumor in chimeras are consistent with those presented here i.e., an influence of bone marrow–derived cells and of the host nonlymphohematopoietic environment on NK cell function can be detected (our unpublished data). Radiosensitive host elements that might influence NK cell development could include marrow stroma, since the normal marrow environment appears to play an important role in NK cell development (23). Although our FCM technique was sensitive to 0.1% contamination of H-2-disparate cells in a mixing study, and adoptive transfer studies from fully allogeneic chimeras in class I–mismatched donor–host combinations suggest that no host–type hematopoietic stem cells survive after lethal irradiation and non-TCD allogeneic BMT (our unpublished data), we have not ruled out the formal possibility that host marrow–derived cells below the level of detection in these assays survive in non-TCD B10→B10.A chimeras. However, we believe it is unlikely that such low levels of microchimerism within the lymphohematopoietic system, if present, would mediate the potent and durable effect we observed in non-TCD B10→B10.A chimeras. It is also formally possible that long-lived radiosensitive host marrow–derived cells residing outside of the lymphohematopoietic system, such as host Langerhans cells that persist in the skin of long-term chimeras (our unpublished data), are the radiosensitive host elements that influence the NK cell repertoire.

Previous studies suggested that the interaction of Ly-49 on NK cells with D4 expressed on target cells provides a negative signal to the NK cell, resulting in failure to kill the target (5). The reduced Ly-49 expression on NK cells of D4-bearing mice, and of chimeras expressing D4 on bone marrow–derived cells or radiosensitive host elements, is consistent with the hypothesis that negative signaling through this interaction with D4 in vivo leads to either destruction of Ly-49+ NK cells or to downregulation of Ly-49 expression. We favor the possibility that Ly-49 expression is downregulated, as we have seen a continuum in the level of Ly-49 expression, with reduced anti-Ly-49 staining intensity in B10→(B10 x B10.A)F1, B10→A/J, and long-term B10→B10.BR chimeras. Even lower, but nevertheless detectable, levels of Ly-49 expression were variably detected on normal B10.A, (B10 x B10.A)F1 and B10.BR NK cells, and in mixed chimeras involving H-2 donors. Thus, it is possible that all of the differences between strains and various types of chimeras reflect varying intensities of Ly-49 expression, rather than the presence or absence of Ly-49+ NK cells.

Ly-49 is one of a family of structurally similar type II integral membrane proteins that is genetically linked to the NKR-P1 family of NK cell–associated genes on mouse chromosome 6 (14, 24, 25). If the interaction of such an NK cell–associated structure, or a combination of such structures on a single NK cell, results in a high affinity interaction with the target cell's class I MHC molecules, then perhaps the negative signal transduced to the NK cell could lead to downregulation of the interacting surface molecule(s) or deletion of that NK cell subset. The lack of Ly-49 expression on NK cells of D4+ mice or chimeras might reflect a very high affinity interaction between D4 and Ly-49, resulting in such a potent negative signal that high levels of Ly-49 expression do not persist. If, on the other hand, the overall affinity of these interactions were intermediate, then the less intense negative signal provided by interactions with self-class I MHC molecules might prevent the killing of autologous targets while allowing that NK cell subset to persist in vivo. These NK cells, on the other hand, would kill targets not expressing the relevant self-class I molecule(s), leading to a pattern of lysis consistent with the "missing self" hypothesis (26–28).

Another NK cell subset marker, recognized by mAb 5E6, is expressed on NK cells that resist engraftment of homozygous H-2b but not of H-2a marrow (29). Thus, interactions of the 5E6 molecule with MHC molecules could be postulated. Ly-49 expression is downregulated in mice expressing the MHC molecules to which it presumably can bind, whereas 5E6+ NK cells are detectable in animals expressing D4. This difference could be explained by our affinity/NK cell subset model. Since 5E6+ NK cells cannot reject H-2b marrow, it could be postulated that the 5E6 molecule is an Ly-49-like recognition structure with intermediate affinity for D4. Expression of 5E6 would ensure that this subset of NK cells did not kill autologous targets in H-2d x H-2d mice, and would not reject homozygous H-2b marrow. In contrast, 5E6− NK cells in F1 mice might rely on expression of an H-2d–binding Ly-49–like molecule to inhibit lysis of autologous targets, and would thus be unable to reject homozygous H-2d marrow. The 5E6+ subset present in H-2a homozygous mice, on the other hand, might express an additional Ly-49–like molecule that interacts with intermediate affinity to an H-2d MHC molecule in order to prevent killing of its autologous, D−negative cells. Thus, phenotypically distinguishable subsets of 5E6+ cells might be present in homozygous versus heterozygous H-2d versus H-2ab F1 mice. Coexpression of a combination of clonally distributed NK cell surface molecules has recently been reported to be associated with the inability of human NK cells to kill targets bearing HLA-Cw3 (4, 30).

Our in vivo studies indicate that NK cell phenotype is most readily controlled by MHC expressed on bone marrow–derived cells, but can also be regulated by radiosensitive host
cells. Thus, NK cells resemble T cells in that both bone marrow-derived and radioresistant host elements determine the presence or absence of cells with certain MHC recognition structures, although bone marrow-derived cells have a more potent ability to ensure that mature cells with host-reactive receptors are not released (31, 32). This negative selection of T cells is mediated by a process of cell death, whereas it remains possible that negative selection of Ly-49-bearing NK cells is not due to permanent deletion but to dynamic regulation of Ly-49 expression by another mechanism, such as decreased transcription, translation or post-translational processing. Studies currently in progress should help to further clarify the mechanism and site of NK cell adaptive differentiation.

We thank Drs. Petter Höglund, Klas Kärre, Lorri A. Lee, David H. Sachs, and Henry J. Winn for critical review of the manuscript. We also thank Drs. Lorri Lee and Yukihiro Tomita for making chimeras prepared with the nonmyeloablative regimen available for our analyses.

This work was supported by National Institute of Allergy and Infectious Diseases grant ROI 31158.

Address correspondence to Dr. Megan Sykes, Transplantation Research Biology Center, MGH-E, Building 149, 13th Street, Boston, MA 02129.

Received for publication 28 January 1992.

References


