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*J Immunol* 2003; 170:5398-5405; doi: 10.4049/jimmunol.170.11.5398

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Yong Zhao, Hideki Ohdan, Jennifer O. Manilay, and Megan Sykes

Alterations in inhibitory receptor expression on NK cells have been detected in mixed allogeneic chimeras and in mosaic MHC class I-expressing transgenic mice. However, it is not known whether or not NK cells are tolerant to host and donor Ags in mixed chimeras. In vitro studies have shown a lack of mutual tolerance of separated donor and host NK cells obtained from mixed chimeras. Using BALB/c→B6 fully MHC-mismatched mixed chimeras, we have now investigated this question in vivo. Neither donor nor host NK cells in mixed chimeras showed evidence for activation, as indicated by expression of B220 and Thy-1.2 on NK cells in chimeric mice at levels similar to those in nonchimeric control mice. Lethally irradiated, established mixed BALB/c→B6 chimeras rejected a low dose of β₂-microglobulin-deficient bone marrow cells (BMC) efficiently but did not reject BALB/c or B6 BMCs. In contrast, similarly conditioned B6 mice rejected both BALB/c and β₂-microglobulin-deficient BMCs. Thus, NK cells were specifically tolerant to the donor and the host in mixed allogeneic chimeras. The similar growth of RMA lymphoma cells in both chimeric and control B6 mice further supports the conclusion that donor BALB/c NK cells are tolerant to B6 Ags in chimeras. Administration of a high dose of exogenous IL-2 could not break NK cell tolerance in chimeric mice, suggesting that NK cell tolerance in chimeras is not due to a lack of activating cytokine. No reduction in the level of expression of the activating receptor Ly-49D, recognizing a donor MHC molecule, was detected among recipient NK cells in mixed chimeras. Thus, the present studies demonstrate that NK cells in mixed chimeras are stably tolerant to both donor and host Ags, by mechanisms that are as yet unexplained. The Journal of Immunology, 2003, 170: 5398–5405.

Natural killer cells, a heterogeneous population of large granular lymphocytes, have been demonstrated to participate in the rejection of virally infected cells, tumor cells, allogeneic and xenogeneic bone marrow cells (BMC), and xenogeneic tissue and organ grafts (1–7). Activation of NK cells is an early event during an immune response and does not involve the expansion of rare Ag-specific cells as is characteristic of adaptive immunity. NK cells mediate “hybrid resistance” in which H-2-homozygous parental BMCs can be rejected by lethally irradiated F₁ hybrid mice (8–10), indicating that compared with T and B cells, NK cells use a distinct approach to recognizing target cells.

It has long been clear that NK cells are tolerant to self Ags, as they fail to attack normal autologous cells, yet frequently reject normal allogeneic, semiallogeneic or class I-deficient cells, including lymphoblasts or hematopoietic stem cells (11–13). The molecular mechanisms by which NK cells distinguish self and nonself are now partially understood. It has been demonstrated that NK cells can recognize MHC molecules expressed on the target cells, and that self-recognition is mediated by inhibitory Ly-49 receptors on mouse NK cells and killer inhibitory receptors in humans (5, 14, 15), as well as CD94/NKG2 receptors in both species (16–18). The missing self hypothesis proposes that target cells become susceptible to NK cell killing when they lack sufficient expression of MHC molecules, the interaction of which with inhibitory NK receptors could prevent NK cell activation. MHC class I-specific stimulatory and inhibitory receptors expressed on NK cells may contribute to the alloreactivity of the self-tolerant mouse NK cells (19–21). Transgenic expression of self MHC class I molecules on target cells significantly inhibited NK cell killing of target cells, supporting the dominant role of NK-inhibitory receptors in allo- or xenoreactive NK cells (21, 22). However, a role for activating Ly-49 receptors in rejection of allogeneic donor BMCs has been recently reported (19).

Altered inhibitory receptor expression on NK cells has been observed in mixed allogeneic chimeric mice and mice with mosaic expression of transgenic allogeneic MHC class I molecules (23–27), demonstrating that natural killer cell-inhibitory receptor expression is regulated by both hematopoietic cells and radioresistant elements. The expression of Ly-49 receptors on NK cells was significantly down-regulated when their corresponding MHC ligands were present during NK cell differentiation. However, the effect of the presence of MHC ligand in the environment is to down-regulate expression of inhibitory receptors recognizing those particular MHC molecules (23–26). This down-regulation does not provide an explanation for NK cell tolerance in mixed chimeras. Indeed, in vitro studies showed that host NK cells from mixed chimeras had the ability to kill donor cells and that donor NK cells could kill recipient targets after culture with IL-2 (25), suggesting that NK cells might not develop mutual tolerance in mixed chimeras. However, mixed chimerism is stable long term, suggesting that donor and host NK cells might be mutually tolerant. This apparent contradiction prompted us...
to investigate NK cell tolerance further in mixed chimeras, using in vivo models.

We used fully MHC-mismatched, mixed allogeneic chimeras prepared with a nonmyeloablative conditioning regimen to examine the tolerant state of NK cells in vivo. Normal NK function was present in chimeric mice, as indicated by the ability to reject β2-microglobulin (β2m)-deficient BMCs after lethal irradiation. No rejection of donor or host BMCs by NK cells in chimeric mice was detected, regardless of whether or not chimeric mice were treated with exogenous IL-2. These results suggest that NK cells maturing in mixed allogeneic chimeric mice are tolerant to donor Ags.

Materials and Methods

Animals

C57BL/6 (B6) (H-2b) and BALB/c (H-2d) mice were purchased from the Frederick Cancer Research Facility (Frederick, MD). MHC class I-deficient (β2m-deficient) C57BL/6J-B2m<sup>tm1Unc</sup> mice (H-2b) were purchased from The Jackson Laboratory (Harbor, ME). All mice were maintained in a specific pathogen-free facility and were housed in microisolator cages containing autoclaved feed, bedding, and acidified water. Animal care was in accordance with the American Association for the Accreditation of Laboratory Animal Care and institutional guidelines.

Preparation of mixed allogeneic chimeras

BALB/c–/B6 mixed chimeras were prepared using a previously described nonmyeloablative conditioning regimen (28). Briefly, 5–8-wk-old C57BL/6 (B6) recipients were treated with depleting doses of anti-CD4 (GK1.5) and anti-CD8 (2.43, respectively) to deplete T cells on day −5 and received 7 Gy of thymic irradiation (TI) and 3 Gy of total body irradiation (TBI) on day 0. Ten million donor BMCs were administered i.v. on day 0. Animals receiving such treatment demonstrate long term mixed chimerism in all hematopoietic lineages, as previously described (28). B6 mice receiving this nonmyeloablative conditioning regimen without donor BALB/c BMC served as nontransplanted, conditioned controls. Chimerism in the bone marrow and spleen was assessed by flow cytometry.

mAbs and flow cytometry (FCM)

The following mAbs were used: FITC-conjugated rat anti-mouse CD3 mAb (145-2C11); FITC-labeled rat anti-mouse pan NK cell mAb (DX5); biotinylated anti-mouse MHC class I; H-2D<sup>b</sup> (34-2-12); biotinylated anti-mouse MHC class I; H-2D<sup>b</sup> (145-2C11); FITC-labeled anti-CD45R/B220 mAb; FITC-labeled anti-Thy-1.1 mAb; PE-conjugated rat IgG2a, biotinylated HOPC-1, and FITC-labeled HOPC-1 were used as nonstaining control mAbs. All mAbs were purchased from BD PharMingen (San Diego, CA). The exception of HOPC-1, which was prepared in our laboratory by protein A-Sepharose purification from ascites and FITC or biotin conjugation by standard procedures.

To determine the chimerism levels, mice were tail bled to obtain white blood cells, as previously described (25). Cells were stained with FITC-labeled rat anti-CD3 or anti-pan NK (DX5) mAb vs biotinylated anti-H-2<sup>d</sup> mAb. Cells were analyzed by two-color flow cytometry using a FACScan (BD Biosciences, San Jose, CA). Nonviable cells were excluded in two-color experiments.

Analyses of Ly-49 marker expression were performed on gated host (DX5<sup>+</sup>CD3<sup>–</sup>CD<sup>–2</sup>-2<sup>–</sup>) or donor (DX5<sup>+</sup>CD3<sup>–</sup>KH95<sup>+</sup>) splenic NK cells obtained at the indicated time of sacrifice. Ly-49D expression was determined using mAb 12A8 kindly provided to us by Dr. L. Mason (National Cancer Institute, Frederick, MD) (30). Because this mAb cross-reacts on Ly-49A and Ly-49C (29), we compared expression of its ligands with that of Ly-49A as detected with mAb YE1/48 (kindly provided by Dr. J. Ortaldo, National Cancer Institute). Purified YE1/48 and 12A8 mAbs were prepared and yeast was conjugated as described (24). 12A8 was used in an indirect stain with a FITC-conjugated rat anti-mouse IgG2a mAb purchased from BD Pharmingen.

Tumor outgrowth

RMA tumor cells were derived from the Rauscher virus-induced lymphoma line RBL-5 of C57BL6 mouse origin (31). They were kindly provided to us by Dr. K. Karre (Karolinska Institute, Stockholm, Sweden). Different doses of RMA suspended in 0.1 ml of PBS were inoculated s.c. into the flanks of chimeric or control B6 mice. Tumor growth was followed by daily palpation. The presence or absence of visible and palpable solid tumor tissue of at least 3 mm in diameter was recorded as tumor.

<sup>125</sup>I-labeled ido-t2-deoxyxuridine ([<sup>125</sup>I]ltdU) NK cell assay

Established mixed chimeric and conditioned control mice were exposed to TBI (10.5 Gy) 6 wk after the original nonmyeloablative conditioning. These mice then received 1 × 10<sup>8</sup> B6, BALB/c, or MHC class I-deficient (β2m-deficient) mouse BMCs i.v. via a lateral tail vein on day 0. In some experiments, mice were also injected i.p. with anti-CD4 and anti-CD8 mAbs (GK1.5 and 2.43, respectively) to deplete T cells on day −5, and other mice received i.p. injection of anti-NK mAbs (PK136 mAb, 0.15 mg) or anti-asialo-GM1 (Wako Chemicals USA, Richmond, VA) on day −2 to deplete either recipient B6 (6) or both recipient B6 and donor BALB/c-derived NK cells in chimeric mice. Some mice received i.p. injections of 50,000 U of recombinant human IL-2 (Chiron Therapeutics, Emeryville, CA) on days −3, −2, and −1. On day 5, 3 μCl of [<sup>125</sup>I]ltdU in 0.5 ml PBS were inoculated i.p. 1 h after 25 μg of 5′-fluoro-2′-deoxyuridine in 0.2 ml PBS were injected i.p. After 20 h, mice were sacrificed, and the radioactivity in the spleen was measured by a gamma counter. In all experiments, chimeric and control mice receiving no BMCs served as irradiation controls, and previously conditioned B6 mice receiving syngeneic BMCs served as positive controls for BMC engraftment.

Adaptive transfer of T cell-depleted splenocytes

T cell-depleted B6 splenocytes (5 × 10<sup>7</sup>) were injected i.v. into lethally irradiated syngeneic B6 or allogeneic BALB/c mice. Four days later, splenocytes from 2–3 mice were collected and stained with PE-labeled anti-NK1.1 mAbs (PK136) and biotinylated 2C11 mAbs vs FITC-labeled anti-CD220, B6, or HOPC-1 mAb. 2C11<sup>+</sup>PK136<sup>+</sup> cells were gated as B6 cells. Similarly, 5 × 10<sup>7</sup> T cell-depleted BALB/c spleenocytes were injected i.v. into lethally irradiated syngeneic BALB/c or allogeneic B6 mice. Four days later, gated BALB/c NK cells (CD3<sup>+</sup>H-2<sup>d</sup>DX5<sup>+</sup>) in splenocytes were assayed by three-color FCM.

Statistical analysis

Student’s t test for comparison of means was used to compare groups. A p value <0.05 was considered to be statistically significant.

Results

Host and donor NK cells do not show evidence of increased activation levels in mixed allogeneic chimeras

During NK cell activation, the expression of a number of markers, including CD220, Thy-1, CD2, LFA-1, CD69, CD45RA, and CD45RO, is up-regulated on mouse and human NK cells (32–38). Markedly up-regulated expression of B220 and Thy-1.2 on B6 NK cells was observed when B6 mice were treated with polyinosinic-cytidyllic acid or NK cells were cultured with IL-2 in vitro (data not shown). Four days after adoptive transfer of B6 splenocytes to lethally irradiated BALB/c mice, B6 NK cells were significantly enlarged and expressed higher levels of B220 and Thy-1.2 in allogeneic BALB/c recipients than in lethally irradiated syngeneic B6 recipients (Fig. 1A). Similar results were observed when BALB/c NK cells were adoptively transferred to B6 recipients (Fig. 1B). These results demonstrate that enlarged cell size and
up-regulated expression of B220 and Thy-1.2 on NK cells may serve as NK activation markers in vivo, consistent with previous in vitro studies (33, 38).

If NK cell tolerance does not develop in mixed allogeneic chimeras, their NK cells might be in a chronic state of activation. To determine whether or not donor- or host-derived NK cells in BALB/c→B6 mixed chimeras were in an activated state, we examined the expression of activating markers on NK cells by three-color FCM. Normal levels of T and NK cells were detected in the peripheral blood of BALB/c BMC-engrafted B6 mice (i.e., mixed chimeras) at 6 wk post-BMT (data not shown). About 60% of T cells, monocytes, and NK cells in the blood of chimeric mice were derived from the BALB/c donor (data not shown). Activation markers on donor and host NK cells were assayed by gating CD3<sup>-</sup>DX5<sup>+</sup> cells as B6-derived NK cells and CD3<sup>+</sup>H-2<sup>dm</sup>DIX<sup>+</sup> cells as BALB/c-derived NK cells, respectively, in chimeric mice (the donor strain, BALB/c, does not express NK1.1<sup>+</sup>, allowing use of this marker to identify host-derived NK cells). As is shown in Fig. 2, no significant differences in the percentages of B220<sup>+</sup> or Thy-1.2<sup>+</sup> cells were detected among B6 NK cells of mixed chimeras compared with conditioned controls. Furthermore, the intensity of B220 and Thy-1.2 expressed on B6 NK cells in chimeric B6 mice was similar to that in conditioned control B6 mice. Likewise, no striking evidence for activation of BALB/c NK cells was detected in mixed chimeric B6 mice compared with control BALB/c mice. No significant changes in NK cell size were detected in chimeric mice compared with control mice (data not shown). These studies suggest that neither host nor donor NK cells in chimeric mice were in a heightened state of activation.

**FIGURE 1.** Up-regulated B220 and Thy-1.2 expression on mouse NK cells after adoptive transfer to allogeneic recipients. a, T cell-depleted B6 splenocytes (5 × 10<sup>7</sup>) were injected i.v. into lethally irradiated syngeneic B6 or allogeneic BALB/c mice. Four days later, gated B6 NK cells (CD3<sup>-</sup> NK1.1<sup>+</sup>) in splenocytes were assayed by three-color FCM, as described in Materials and Methods. b, T cell-depleted BALB/c splenocytes (5 × 10<sup>7</sup>) were injected i.v. into lethally irradiated syngeneic BALB/c or allogeneic B6 mice. Four days later, gated BALB/c NK cells (CD3<sup>+</sup>H-2<sup>dm</sup>DIX<sup>+</sup>) splenocytes were assayed by three-color FCM, as described in Materials and Methods. Pooled splenocytes from three mice in each group are shown. Values are the results of one of two similar experiments.

**FIGURE 2.** No increase in B220 and Thy-1.2 expression on host or donor NK cells in mixed allogeneic chimeras. Six weeks after BALB/c BMT to B6 mice conditioned with 7 Gy TI, 3Gy TBI, and T cell-depleting mAbs, gated B6 (CD3<sup>-</sup> NK1.1<sup>+</sup>) or BALB/c (CD3<sup>+</sup>H-2<sup>dm</sup>DIX<sup>+</sup>) NK cells in splenocytes from chimeric B6 mice and simultaneous, similarly conditioned control B6 or BALB/c mice were assayed by three-color FCM, as described in Materials and Methods. Each symbol represents one animal. No significant difference was observed between the groups.

**NK cells in lethally irradiated chimeric mice fail to reject low doses of host and donor BMCs, but reject β<sub>2m</sub>-/− BMCs**

NK cells are responsible for the rapid rejection of bone marrow cell allografts in lethally irradiated and T cell-deficient mice. The standard assay for in vivo determination of NK cell function is the rejection of a low dose of allogeneic BMCs injected into lethally irradiated recipients. In this assay, engraftment is detected by [125<sup>I</sup>]UdR incorporation in spleens 5 days later. Six weeks after bone marrow transplantation, mixed chimeric B6 mice showed normal levels of T and NK cells in the peripheral blood. The percentages of donor-derived T cells, NK cells and granulocytes in the WBCs of 20 representative chimeras are shown in Table I. These mixed chimeric mice and the conditioned control mice (i.e., mice that received nonmyeloablative conditioning without BMCs) were then treated with lethal irradiation and received an i.v. injection containing 10<sup>6</sup> B6, BALB/c, or β<sub>2m</sub>-deficient BMCs. Five days later, the extent of donor cell survival and proliferation was determined by a standard [125<sup>I</sup>]UdR incorporation assay. As is shown in Fig. 3, conditioned control B6 mice accepted B6 BMCs and rejected BALB/c and β<sub>2m</sub>-deficient BMCs. The rejection of allogeneic BMCs in this model was mediated by NK cells, as indicated by the increased engraftment of BALB/c BMCs in NK cell-depleted B6 mice. Mixed chimeric B6 mice also rejected β<sub>2m</sub>-deficient mouse BMCs, indicating that they have functional NK cells.
FIGURE 3. NK cells in chimeric B6 mice do not reject BALB/c or B6 BMCs but reject βm-deficient BMCs. One million B6, BALB/c, or βm-deficient BMCs were injected i.v. into lethally irradiated chimeric and conditioned control B6 mice (6 wk after conditioning). Recipient mice were untreated or received total NK cell depletion with anti-asialo-GM1 or host (B6) NK cell depletion with anti-NK1.1 mAb (PK136) before irradiation. The injected BMC proliferation was measured using standard [3H]UdR incorporation in spleens. Each symbol represents an individual mouse. Data from two independent experiments are combined. *** p < 0.001, compared with the indicated groups; NS, no statistical difference between the indicated groups.

Tolerance of NK cells to donor and host in mixed chimeric mice

Our previous studies showed that separated host NK cells from mixed allogeneic chimeras are able to kill donor lymphoblasts after culture with IL-2 for several days (25). In view of the above in vivo data indicating that host NK cells are tolerant of the donor in mixed chimeras, we considered the possibility that the addition of exogenous IL-2 in vitro broke NK cell tolerance. To test the possibility that this could occur in vivo, we evaluated NK cell tolerance in mixed chimeric mice after administration of a high dose of exogenous IL-2. Mixed chimerism was established by injection of 10⁷ BALB/c BMCs after conditioning with 3 Gy of TBI, 7 Gy of TIL, and T cell-depleting mAbs in B6 mice. These animals showed normal levels of T and NK cells in the periphery, and again, ~60% of these cells (63.8 ± 23.3%, n = 45) were derived from the BALB/c donor 6 wk after BMT. These chimeric mice then received injections of a high dose of recombinant human IL-2 (50,000 U) on each of days −3, −2, and −1, and lethal TBI on day 0. One million B6, BALB/c, or βm-deficient BMCs were injected i.v. on day 0. Five days later, the extent of donor cell survival and proliferation was determined by [3H]UdR incorporation in spleens. Results showed no significant rejection of B6 or BALB/c BMC in chimeric B6 mice, regardless of whether or not they received injection of exogenous IL-2 or depleting anti-NK cell mAb (data not shown). Again, chimeric B6 mice rejected βm-deficient BMCs as effectively as conditioned B6 control mice. These studies showed that NK cells in mixed chimeras are specifically tolerant of
the donor but have a normal capacity to reject class I-deficient bone marrow cells, even when exogenous IL-2 is administered.

Anti-H-2d activating receptor Ly-49D does not explain tolerance of host NK cells to the donor

We have previously obtained evidence that up-regulation of inhibitory Ly-49 receptors does not occur in response to induction of mixed chimerism and is unlikely to explain tolerance to donor and host Ags (23–25). Recent studies have identified Ly-49D as an inhibitory Ly-49 receptor that interacts with the adaptor molecule DAP12 to provide an activating signal to NK cells (30, 40, 41) upon recognition of H-2Dd (42). We therefore considered the possibility that recipient B6 NK cells might be tolerized to the donor by down-regulation of this activating receptor that recognizes donor class I MHC. Mixed chimeras were prepared in B6 recipients as above but received varying doses of BALB/c marrow (5, 10, or 20 × 10⁶ cells) to achieve a wide range of chimerism levels. The effects of levels of chimerism on inhibitory receptor expression in the groups of mice has been previously reported for these same experiments (24) and demonstrated an inverse relationship between the percentage of BALB/c hematopoietic cells and the intensity of expression of inhibitory receptors recognizing H-2d, including Ly-49G2 and Ly-49A. However, no relationship between the level of chimerism and the intensity of staining with mAb 12A8, which recognizes both Ly-49A and Ly-49D, was detected in the same animals at the same time points (5 and 6–6.5 wk post-BMT) in two experiments. Overall, the chimeras (n = 18 in one of two similar experiments analyzed at 5 wk) showed a significantly reduced intensity (average ± SEM of median fluorescence intensity [MFI], 139 ± 32) of staining with mAb against the inhibitory receptor Ly-49A on B6 NK cells compared with similarly conditioned, simultaneous non-BMT B6 controls (n = 4; MFI 594 ± 91; p < 0.05; Fig. 5). In contrast, no difference between the chimeras and the controls was detected in the intensity of staining with mAb 12A8, which recognizes Ly-49A and Ly-49D (average ± SEM; MFI 63 ± 18 for chimeras vs 71 ± 24 for controls; p = 0.6, Fig. 5). These results indicate that Ly-49D levels on B6 NK cells were not decreased in chimeras compared with controls. In fact, the similar levels of staining with mAb 12A8, combined with the reduced expression of Ly-49A in the chimeras compared with controls, suggests that Ly-49D expression might have been increased on B6 NK cells in the chimeras compared with controls. We also detected no difference in the percentage of Ly-49D⁺ cells among B6 NK cells in chimeras and controls and saw no correlation between the level of chimerism and the intensity of staining with mAb 12A8 (r = 0.4, with a positive slope, correlating percentage of donor NK cells in spleen and intensity of 12A8 staining; data not shown). Thus, down-regulation of Ly-49D expression does not appear to be a mechanism of tolerance of recipient NK cells to the donor in these mixed chimeras.

Discussion

Very early studies, including the demonstrations that F₁ to parent chimeras lacked hybrid resistance (43) and that hybrid resistance can be abrogated by neonatal tolerance induction with parental strain cell transplantation (44), suggested that NK cells could adapt their specificity to the environment in which they develop. However, the mechanisms of this adaptation are not well understood. It has been proposed that multiple mechanisms, including expression of self-specific inhibitory receptors, modulation of the cell surface levels of MHC-specific inhibitory or activatory receptors, and energy may be involved in NK cell tolerance (45). It has been reported that the presence of Ly-49D⁺ NK cells in H-2b/d F₁ hybrid mice is responsible for the rejection of H-2b/d BMCs, indicating that loss of Ly-49D expression in H-2b/d⁺ mice is not a mechanism of self-tolerance (19). Studies have suggested that self-tolerance of Ly-49D⁺ NK cells may be achieved through coexpression...
of a sufficient level of self-specific inhibitory receptors (19), but a similar mechanism has not been demonstrated for allogeneic tolerance.

In contrast to our detailed understanding of the mechanisms of T cell tolerance in mixed allogeneic chimeras (46–48), little is known about NK cell tolerance in this model. The stable state of mixed hemopoietic chimerism observed in such animals could indicate that donor and host NK cells are tolerant of one another. In contrast, because NK cells do not have a strong ability to destroy the most primitive pluripotent stem cells (49) but clearly have the ability to kill committed hemopoietic progenitor cells (10, 50, 51), a failure of mutual tolerance of donor and host NK cells could result in a state of chronic rejection of both donor and host hemopoietic progenitors, which might result in a stable state of mixed chimerism, perhaps with some degree of hemopoietic failure. Although we have not observed evidence of hemopoietic failure in long term chimeras, we did observe a long term increase in donor chimerism in mice receiving chronic treatment with NK cell-depleting mAbs to deplete both donor and host NK cells in the B10→B10.A chimera model (49, 52). However, injection of anti-NK mAb (PK136) to deplete both donor and host NK and NKT cells simultaneously made it difficult to determine the role of host NK cells in the decrease of donor chimerism. Furthermore, because primitive pluripotent stem cells are relatively resistant to NK cell-mediated rejection, they could continuously make more progenitors to compensate for NK cell-mediated destruction if it occurred, so that long term chimerism might not provide a direct indication of NK cell-mediated rejection. Thus, a standard short term in vivo NK function study using a low dose of donor BMC is essential to address whether or not host NK cells are tolerant to donor Ags in chimeras.

One mechanism by which individual NK cells in mixed chimeras might be rendered tolerant to donor and host would be for each NK cell to express inhibitory receptors recognizing donor and host MHC ligands. The expression of inhibitory receptors on NK cells in mixed allogeneic chimeric mice has been shown to be altered by the presence of an additional MHC haplotype expressed on either hemopoietic or nonhemopoietic cells (23–25). The presence of MHC ligand on cells other than the NK cell itself can influence the expression of Ly-49 receptors. For example, Ly-49A expression on D\(^{a}\)\(^{-}\) NK cells was influenced by D\(^{a}\) expression on both hemopoietic and radioresistant (nonhemopoietic) host elements in mixed and fully allogeneic bone marrow chimeras or mice with mosaic expression of a D\(^{a}\) transgene (5, 23–25, 53). Thus, the presence of MHC ligand in the environment, and not necessarily on the same cell, is sufficient to induce inhibitory Ly-49A receptor down-regulation, and the level of down-regulation varies with the amount of MHC ligand in the environment (23, 24). However, the functional significance of the changed NK inhibitory receptors is not well understood. These studies showed no evidence for modulation of inhibitory receptors as a mechanism of tolerance (25), given that no increase in the proportion of NK cells expressing receptors for both donor and host MHC ligands was observed.

Using a fully MHC-mismatched BALB/c→B6 mixed chimeric mouse model (24, 25), in which 10\(^7\) BALB/c BMCs were transplanted to B6 mice receiving nonmyeloablative conditioning and in which ~60% BALB/c-derived cells were detected in all lineages in the peripheral blood of chimeric B6 mice 6 wk post-BMT, we have now investigated the NK cell tolerant state in vivo. The results of these studies indicate that both host and donor NK cells in mixed chimeras are specifically tolerant to both donor and host Ags in vivo. First, no evidence of constitutive NK cell activation was detected in chimeric mice compared with nonchimeric control mice (Fig. 2). Secondly, when a standard NK function assay was performed in vivo, lethally irradiated chimeric mice rejected β\(^{m}\)-deficient BMCs as efficiently as control mice, indicating intact NK cell function in chimeric mice. However, lethally irradiated mixed chimeras showed similar levels of engraftment of both donor and host BMCs, indicating a failure of NK cells to reject a low dose of donor or host hemopoietic cells (Fig. 3). Thirdly, if donor NK cells were not tolerant of the recipient, recipient syngeneic tumor growth should be significantly delayed, due to donor NK cell-mediated rejection. Similar RMA tumor growth in chimeric and control B6 mice indicated no additional tumor killing mediated by allogeneic donor bone marrow cell-derived NK cells in chimeric mice (Fig. 4).

Despite this compelling evidence that NK cells in mixed allogeneic chimeras are mutually tolerant of one another, NK cells from mixed allogeneic chimeras or mosaic transgenic mice can be activated in vitro by culture in IL-2 to mediate killing in vitro of cells bearing MHC phenotypes to which they show tolerance in vivo (25, 53). These results suggest that NK cell tolerance does not occur via deletion of the potentially alloreactive NK cell subset(s) and instead suggest that anergy or failure of activation in vivo may play a role in NK cell tolerance in mixed allogeneic chimeras and mosaic transgenic mice. Our present in vivo studies show that NK cells failed to reject donor BMCs in BALB/c→B6 chimeric mice even after administration of a high dose of exogenous IL-2 cytokine. These data may argue against the concept that NK cells in chimeric mice fail to reject cells of the other MHC type due to a failure to be activated in vivo. However, additional studies would be needed to definitively rule out this possibility.

The reasons for the inconsistency between the in vivo and in vitro results are therefore not clear. That different target cells were used in vivo and in vitro may be one of the reasons. In in vitro assays, Con A-stimulated lymphoblasts were used as targets, and the MHC expression and the artificial conditions may not reflect the in vivo conditions. In addition, the in vitro NK function assays were performed by culturing purified donor or host NK cells with IL-2 for 4 days (25). Studies in mosaic transgenic mice (DL6) (53) showed that D\(^{a}\)/L\(^{d}\)\(^{-}\) and D\(^{a}\)/L\(^{d}\)\(^{+}\) NK cells were mutually tolerant to the opposite phenotype in vivo and in vitro when unseparated mosaic splenocytes were used as effector cells, whereas D\(^{a}\)/L\(^{d}\)\(^{+}\) NK cells killed the D\(^{a}\)/L\(^{d}\)\(^{-}\) targets when separated D\(^{a}\)/L\(^{d}\)\(^{+}\) splenocytes from mosaic transgenic mice were used as effector cells and cultured for 1 or 4 days. Thus, the tolerant state of NK cells may rely on the continued presence of the relevant Ags in their environment. However, this does not explain the results in mixed chimeras, because the lack of NK cell tolerance in vitro was seen regardless of whether or not the relevant allogeneic cell type was present during the period of in vitro culture of NK cells (25).

Data presented here showed no down-regulation of the H-2\(^{d}\) recognizing activating receptor Ly-49D among B6 NK cells of BALB/c→B6 chimeras compared with controls, arguing against down-regulation of activating receptors as a mechanism of NK cell tolerance. Ly-49D-expressing NK cells of B6 mice have been shown to be required for the rejection of H-2\(^{d}\) \times \(^{b}\) marrow (20). Thus, for tolerance to be present among host NK cells in our mixed chimeras, some other mechanism must prevent this positive signal on recipient NK cells from causing rejection of the donor hemopoietic cells. Our data are in contrast to those of Wilk et al. (54), who detected a reduction in the percentage of Ly-49D\(^{+}\) NK cells and a reduction in the level of expression of this molecule among H-2\(^{d}\) NK cells in full chimeras prepared with lethal irradiation in the H-2\(^{d}\)\→\→H-2\(^{d}\) combination. These authors also detected tolerance to the donor by in vitro cytotoxicity assays (54), also in contrast to our own results in mixed chimeras (25). Collectively, these
results suggest that the mechanisms of NK cell tolerance in full allogeneic chimeras, in which an allogeneic MHC is expressed only by nonhemopoietic cells, may differ from those in host-derived NK cells in mixed chimeras, in which the allogeneic MHC is expressed only by hemopoietic cells. It might also differ from those of donor-derived NK cells in mixed chimeras, in which the allogeneic MHC is expressed by both hemopoietic and nonhemopoietic cells.

In summary, the present data indicate that NK cells in mixed allogeneic chimeric mice are specifically tolerant to both donor and host Ags and that this tolerance is unlikely to be due to a lack of activation in vivo or to down-regulation of activating Ly-49 receptors. Further studies are needed to further evaluate the mechanism of NK cell tolerance in these mice.

Acknowledgments

We thank Drs. Toshi Kawahara and Paul Russell helpful review of the manuscript. We also thank Orlando Moreno for outstanding animal husbandry and Robin C. Laber for expert assistance in preparing the manuscript.

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