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Prenatal Allospecific NK Cell Tolerance Hinges on Instructive Allorecognition through the Activating Receptor during Development

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Little is known about how the prenatal interaction between NK cells and alloantigens shapes the developing NK cell repertoire toward tolerance or immunity. Specifically, the effect on NK cell education arising from developmental corecognition of alloantigens by activating and inhibitory receptors with shared specificity is uncharacterized. Using a murine prenatal transplantation model, we examined the manner in which this seemingly conflicting input affects NK cell licensing and repertoire formation in mixed hematopoietic chimeras. We found that prenatal NK cell tolerance arose from the elimination of phenotypically hostile NK cells that express an allospecific activating receptor without coexpressing any allospecific inhibitory receptors. Importantly, the checkpoint for the system appeared to occur centrally within the bone marrow during the final stage of NK cell maturation and hinged on the instructive recognition of allogeneic ligand by the activating receptor rather than through the inhibitory receptor as classically proposed. Residual nondeleted hostile NK cells expressing only the activating receptor exhibited an immature, anergic phenotype, but retained the capacity to upregulate inhibitory receptor expression in peripheral sites. However, the potential for this adaptive change to occur was lost in developmentally mature chimeras. Collectively, these findings illuminate the intrinsic process in which developmental allorecognition through the activating receptor regulates the emergence of durable NK cell tolerance and establishes a new paradigm to fundamentally guide future investigations of prenatal NK cell–allospecific education. The Journal of Immunology, 2015, 195: 1506–1516.

The prenatal exposure to alloantigens is an important feature of immunologic development in eutherian mammals. Both innate and adaptive components of the fetal immune system have evolved to temper the hazards of alloimmunity or autoimmunity with the emergence of prenatal self-tolerance. Since the seminal work of Owen (1), Burnet et al. (2), and Medawar and colleagues (3), much has been written about the origins of self-tolerance; however, few studies have examined the mechanisms or significance of prenatal NK cell tolerance.

Current evidence suggests that NK cell self-tolerance results from the interaction of inhibitory NK cell receptors with their environment, resulting in a mature NK cell repertoire that is fine-tuned to self-MHC class I expression (4–7). With the gain or loss of either cognate (8–10) or noncognate MHC class I self-Ags (11), significant changes occur within the NK cell compartment that result in self-tolerance, but maintain otherwise normal immunity. Evidence also exists for the instructive influence of NK cell–activating receptor interactions with environmental ligands in altering the phenotype and function of the NK cell repertoire (12–14). However, animal models in which the target ligand is ubiquitously expressed throughout development do not adequately emulate the more complex setting of in utero hematopoietic cellular transplantation (IUHCT) or perhaps an encounter between a developing fetal NK cell and a maternal cell during naturally occurring maternal–fetal cellular trafficking (15). More specifically, these studies do not permit fine modulation of the level of ligand exposure to multiple inhibitory or activating receptors, which is logically the most significant parameter in determining prenatal tolerance or alternatively immunization.

Indeed, we previously confirmed that a minimum level of circulating chimerism is necessary to induce durable NK cell tolerance to prenatally transplanted allogeneic hematopoietic cells (16). Recipients with high chimerism levels established and maintained stable engraftment and exhibited donor-specific NK cell tolerance. Conversely, recipients with low chimerism levels displayed NK cell–dependent graft rejection. The essence of this quantitative model for NK cell education is that allospecific tolerance requires exposure to a critical level of ligand exposure during development—a chimerism threshold. In those experiments, host NK cells from chimeric mice naturally expressed both activating and inhibitory Ly49 receptors that were specific for the donor MHC class I ligands. Following preimmune transplantation to an otherwise unmanipulated allogeneic fetal host, direct trans- recognition of donor cells by activating and inhibitory receptors most likely played a dominant role in the education of host NK cells, although indirect or even cis recognition by inhibitory receptors resulting from MHC transfer.
may have had an important role in the education of host NK cells (17–20). It may be speculated that a threshold level of circulating chimerism was critical to each of these mechanisms. In any case, current models of NK cell education do not explain how contradictory activating and inhibitory input signals are reconciled during NK cell education to result in rejection or tolerance. In this study, prenatal allogeneic NK cell tolerance was examined in prenatal chimeras. The present findings illustrate a leading role for the instructive allore cognition by the activating receptor during development in determining the mature NK cell repertoire and the functional competence of phenotypically distinct NK cell subsets in prenatal hematopoietic chimeras.

**Materials and Methods**

**Animals**

Breeding stock of B6Ly5.2 (H2b, Ly5.2) and B6Ly5.1 (H2b, Ly5.1; The Jackson Laboratory, Bar Harbor, ME) and BALB/c (H2d, Ly5.2; Charles River Laboratories, Wilmington, MA) mice were bred in our colony in the Cincinnati Children’s Research Foundation. All experimental protocols were approved by the Institutional Animal Care and Use Committee and in compliance with the Department of Health Guide for the Care and Use of Laboratory Animals.

**In utero transplantation**

In utero transplants were performed as previously described (16) and illustrated in Fig. 1A. Briefly, BALB/c fetal liver light-density mononuclear cells (LDMCs) were harvested from donor fetuses at embryonic day 14 (day of plug = d0) using Ficoll gradient separation (Histopaque 1077, Sigma-Aldrich, St. Louis, MO). Under isoflurane anesthesia, a midline laparotomy was made in the recipient, and the uterus was exposed. Recipient B6 fetuses were injected through the translucent uterine wall with a 5 μL suspension of LDMCs using a 100 μm beveled glass micropipette. The uterus was returned and the abdomen closed with absorbable suture. Pregnant dams were housed individually and given buprenorphine i.p. until parturition. The uterus was returned and the abdomen closed with absorbable suture. The uteri were returned and the abdomen closed with absorbable suture. The pups were weaned at 3 wk of age at which initial level of donor chimerism was determined, as outlined below.

**mAbs and flow cytometry**

LDMCs were isolated from submandibular blood samples initially at weaning (3 wk) to determine early chimerism level and at subsequent time points according to experimental protocol. The following mAbs were purchased from eBioscience or BD Pharmingen (San Diego, CA), unless otherwise specified: CD45 anti-Ly5 (30-F11), H-2Kd (SF1-1.1), CD3ε (145-2C11), NK1.1 (PK136), DX5 (DX5), Ly49A (YE1/48; BioLegend, San Diego, CA), Ly49C (40C-311; provided by W. Yokoyama, St. Louis, MO), Ly49D (4E5), Ly49I (YLI-90), Ly49G2 (CWY-3), Ly49H (3D10), Ly49I (5E6), CD11b (M170), CD27 (LG.7F9), NKG2A (16A11), Ly49F (HBF-719; Novus Biologicals, Littleton, CO), IFN-γ (XM1G2.1), and BrdU (PRB-1). Dead cells were excluded using Hoechst 33342. Where applicable, when chimerism was determined, the percentage of non-erythroid donor chimerism was calculated as the percentage of H-2Kd+ cells within the total (donor + host) CD45+ gate. Samples were analyzed on a BD LSR II and acquired using BD FACSDiva software (both from BD Biosciences, San Diego, CA). Data and figures were prepared using FlowJo (Tree Star, Ashland, OR).

**In vivo NK cell subset depletion**

Rejector animals with low-level chimerism (peripheral blood chimerism ranging from 0.9 to 1.7%) were subjected to in vivo depletion of Ly49D or Ly49C/NK cells using monthly i.p. injections of purified 4E5 mAb or 5E6 mAb, respectively, at 100 μg/dose from 3 to 19 wk of age. Chimerism and the frequency of Ly49D or Ly49C/NK cells were assessed weekly in the peripheral blood of treated recipients and littermate controls, including immediately prior to the monthly redosing of the Ab to ensure >99% depletion of the NK cell subset throughout the study period.

**Intracellular cytokine assay**

Twelve-well plates were coated overnight with 10 μg/ml anti-Ly49D and anti-NK1.1 mAb. Plates were washed with sterile PBS. LDMCs were separated from freshly harvested splenocytes, washed twice, and cultured at a density of 10^6 cells into each of the coated wells or uncoated (negative control) and PMA/ionomycin (positive control) wells. Following a 4-h incubation at 37°C in the presence of brefeldin A (BD Biosciences), the splenocytes were harvested and extracellular staining was performed, as described above, followed by intracellular staining using a BD Cytofix/Cytoperm kit (BD Biosciences). Because Abs against NK1.1 were used for stimulation, the percentages of NK cells that expressed IFN-γ were determined by gating on CD3 DX5+ lymphocytes.

**In vivo BrdU assay**

Engrafter mice (peripheral blood chimerism >1.8%) and age-matched naive controls were selected at 2, 4, 7, and 10 wk of age. Animals were injected with 200 μg BrdU (Sigma-Aldrich) twice daily for 3 d via i.p. injection. On the fourth day, spleen was harvested from both groups and LDMCs were prepared as above. Extracellular staining was first achieved, and the cells were then washed twice in preparation for intracellular staining. A total of 10^6 cells was incubated in 1 ml 10% DMSO and 90% PBS (both from Sigma-Aldrich) and frozen overnight at −80°C. The cells were then thawed, permeabilized, and incubated with DNase 1 at 37°C for 1 h prior to washing and staining with BrdU FITC (PRB-1).

**Adaptive transfer**

Splenic LDMCs were isolated from naive B6Ly5.1 mice at either 3 wk (immature) or 7 wk (mature), Ab labeled, and sorted on a FACSaria (BD Biosciences) isolating CD3- ‘NK1.1’Ly49D+AFG+ NK cells (Ly49D+ NK cells that do not coexpress Ly49A, Ly49F, or Ly49G; hostile NK cells). A total of 3–4 × 10^6 cells/mouse of purified hostile NK cells was transferred to 7-wk-old engrafter B6Ly5.2 and age-matched naive B6Ly5.2 mice via retro-orbital injection. The transferred NK cells were followed serially in the peripheral blood of the recipients, and the percentage of Ly49D+AFG+ (Ly49D+ NK cells that coexpress any combination of Ly49A, Ly49F, or Ly49G) NK cells was calculated among the transferred Ly5.1+ cells.

**Statistical analysis**

Statistical comparisons were performed using a two-tailed Student t test assuming unequal variances. Engraftment rates for the in vivo depletion experiments were compared using the Fisher’s exact test. All statistical calculations were performed using Excel software (Microsoft, Redmond, WA).

**Results**

Host NK cells expressing donor-specific activating receptors are responsible for graft rejection following IUHCT

B6 mice express high levels of the Ly49D-activating receptor on ~55% of their mature NK cells, although no self-ligand has been identified. This receptor binds to the H-2Dd MHC class I ligand that is expressed on the surface of all BALB/c cells, and Ly49D+ cells have been shown to be responsible for rejection of BALB/c (H2d) marrow transplants by adult B6 (H2b) mice (21, 22). To determine whether this mechanism is consistent in IUHCT, we employed a prenatal BALB/c→B6 allotransplantation model (Fig. 1A) in which tolerance or rejection is predicted by the level of early peripheral blood chimerism (16). As shown (Fig. 1B), a chimerism level >1.8% at 3 wk of age (chimerism threshold) resulted in stable long-term engraftment and donor-specific tolerance in 100% of the B6 recipients (engrafters). Conversely, a chimerism level <1.8% resulted in universal graft rejection in these recipients (rejecters).

To determine whether host NK cells expressing an allospecific activating receptor were responsible for the graft rejection, Ly49D+ (4E5) NK cells were selectively depleted from the rejector chimeras (0.9–1.7% chimerism), and chimerism and Ly49D+ NK cell frequency were followed at serial time points (Fig. 1C). Intravenous injections with 4E5 mAb were initiated at 3 wk of age immediately following baseline measurements. Effective elimination of Ly49D+ NK cells (>99%) was achieved by 4 wk of age and maintained through 20 wk, after which the Ly49D+ NK cells returned (Fig. 1C, Supplemental Fig. 1). As shown, the selective in vivo depletion of Ly49D+ cells preserved engraftment in all of
the rejecter mice when compared with the untreated or the Ly49C/I-depleted (5E6) rejecter controls (Fig. 1D, 1E). To further demonstrate that the rejection was specifically mediated by the Ly49D+ NK cells, the Ab injections were discontinued at 19 wk of age and the Ly49D+ NK cells were allowed to return. Graft rejection was seen in 8 of the 12 previously depleted mice soon after the Ly49D+ NK cells reappeared. However, 4 of the 12 mice maintained their engraftment despite the return of an almost normal frequency of Ly49D+ NK cells. Interestingly, the chimerism level drifted above the threshold level of 1.8% in each of these mice during the Ly49D-depletion phase (Fig. 1F).

**Host NK cells expressing donor-specific activating receptors persist despite stable prenatal engraftment**

Because Ly49D+ NK cells are responsible for rejection in BALB/c→B6 rejecter mice, we reasoned that they should be eliminated
in engrafter mice in which long-term engraftment is predictably observed. Surprisingly, Ly49D⁺ NK cells persisted in the peripheral blood of engrafter mice at the earliest time point, but at a reduced frequency when compared with naive controls (Fig. 2A). Approximately 40% of NK cells in these tolerant hosts continue to express Ly49D despite their potential for donor reactivity. Similar changes were also present in bone marrow, raising the possibility that the frequency of Ly49D⁺ NK cells had been altered during NK cell development (Fig. 2B) (23). No change was observed in the frequency of NK cells expressing the irrelevant activating receptor Ly49H provided that these cells did not coexpress Ly49D (Fig. 2C).

**NK cells expressing a donor-specific activating receptor without coexpressing a relevant inhibitory receptor are eliminated during NK cell maturation in engrafter mice**

The persistence of ostensibly alloreactive Ly49D⁺ NK cells in engrafter mice may result from the selection of developing Ly49D⁺ NK cells that coexpress donor-specific inhibitory receptors. In the context of BALB/c→B6 prenatal chimeras, each of the potentially donor-reactive host Ly49D⁺ NK cells would be expected to coexpress at least one inhibitory receptor that is specific for the BALB/c MHC class I ligands (e.g., Ly49A, Ly49F, or Ly49G) to remain tolerant. For simplification, these friendly phenotypes will be clustered and described as Ly49D⁺AFG⁺ NK cells. Conversely, host NK cells that do not express a donor-specific inhibitory receptor (Ly49D⁺AFG⁻) are considered hostile due to the unopposed activating signals and should not propagate through the selection process.

In support of a selection process, the frequency of friendly NK cells in peripheral blood was found to be slightly higher (40.3 ± 5.9% versus 30.5 ± 3.6%), whereas the frequency of hostile NK cells was dramatically lower (4.5 ± 1.3% versus 26.8 ± 2.1%) in engrafter mice when compared with naive controls (Fig. 3A, 3B). To gauge the kinetics of NK cell selection relative to the critical phases of NK cell maturation (24), a selection ratio was calculated as the frequency of friendly:hostile phenotypes. A ratio >>1.4 (average ratio in naive controls) would indicate a selective expansion of the friendly Ly49D⁺AFG⁺ subset and/or a selective reduction of the hostile Ly49D⁺AFG⁻ subset had occurred. As summarized, a 4-fold higher selection ratio had already developed in engrafter mice by 3 wk of age, reaching a plateau of ~10-fold over the subsequent weeks as Ly49D receptor expression matured, whereas the average selection ratio in naive control mice remained consistently equal to ~1.4 (Fig. 3C). Furthermore, the changes in the selection ratio required instructive input from the Ly49D-activating receptor as an analysis of inhibitory receptor coexpression in the Ly49D⁻ NK cells showed no differences between the engrafters and controls (Fig. 3C).

Lastly, a detailed analysis of multiple sites of NK cell lymphopoiesis reveals that engraver mice exhibited an overall decrease in total NK cell number in the developmentally immature environment of the bone marrow as well as among the more mature NK cells in the spleen and the peripheral blood (Fig. 3D). In each site, the magnitude of this reduction was proportionate to the loss of hostile NK cells.

**A low frequency of functionally anergic hostile NK cells persists in stable chimeras**

Although their frequency was greatly diminished, phenotypically hostile NK cells consistently accounted for ~4% of all mature engrafter NK cells (Fig. 3B). This may have resulted from the coexpression of Ly49C, Ly49I, NKG2A, or other unknown inhibitory receptors that exhibit weak binding with both donor and host ligands, thereby permitting these cells to remain tolerant (25, 26). However, the coexpression of any Ly49C, Ly49I, or NKG2A (CIN⁺) was similar between engraver and control NK cells (Supplemental Fig. 2). Furthermore, ~20% of the residual Ly49D⁺ AFG⁻ hostile NK cells in engrafter mice did not express any Ly49C, Ly49I, or NKG2A (CIN⁻), which does not explain the persistence of these cells in engrafter mice (Supplemental Fig. 2).

**FIGURE 2.** Donor-reactive Ly49D⁺ NK cells persist at a reduced frequency in engrafter mice. (A) Ly49D⁺ NK cells persist at a lower frequency among the circulating pool of gated CD₃⁺NK1.1⁺ lymphocytes in blood over time. (B) Decreased frequency of Ly49D⁺ cells is consistent in blood, spleen, and bone marrow of engrafter mice. (C) The frequency of NK cells expressing the irrelevant activating receptor, Ly49H, is unchanged unless Ly49D is coexpressed. Data represent mean frequency ± SD for at least five separate animals in either group at each time point. *p < 0.05.
Alternatively, in the absence of a strong inhibitory signal, hostile NK cells may have persisted in the engrafter mice in a hyporesponsive state, as has been shown for NK cells lacking self-specific inhibitory receptors in naive B6 mice (27–29). In support of this hypothesis, hostile NK cells from engrafter mice were markedly hyporesponsive relative to friendly NK cells following stimulation with plate-bound Ly49D or NK1.1 mAb (Fig. 4A) using an established in vitro assay for NK cell responsiveness (30). This was in sharp contrast to hostile NK cells in naive control mice, which were slightly more responsive than friendly NK cells in the same assays (Fig. 4A). Additionally, the response to calcium flux (PMA and ionomycin) was maintained, confirming that the hostile NK cells retained the capacity to produce IFN-γ.

Lastly, Ly49D+ NK cells coexpressing any combination of Ly49A, Ly49F, or Ly49G in engrafter mice exhibited normal responsiveness, indicating that expression of at least one inhibitory receptor for the donor prevented the development of anergy (Fig. 4B). These observations demonstrate that the selective hyporesponsiveness of hostile NK cells is at least partially regulated by cell-intrinsic mechanisms that result in donor-specific tolerance.

Immature hostile NK cells accumulate in the hematopoietic organs of prenatal chimeras and exhibit a low-avidity phenotype

The selective hyporesponsiveness of hostile NK cells may have resulted from changes in their maturation or their avidity for the donor ligands similar to self-reactive T and B cells (31, 32). To examine this possibility, we adopted the CD27/CD11b paradigm of NK cell maturation, which dissects NK cells into three distinct subsets of maturation, as follows: CD27hiCD11b* (R1); CD27hiCD11bhi (R2); CD27loCD11bhi (R3) (33, 34). In examining the maturation of engrafter NK cells, we noted an overall shift of hostile Ly49D+ AFG2 NK cells toward a higher frequency of the R1 phenotype and lower frequencies

**FIGURE 3.** Donor-specific tolerance in prenatal chimeras arises from the elimination of hostile phenotypes and the propagation of friendly phenotypes. (A and B) Comparison of donor-specific inhibitory receptor coexpression by Ly49D+ NK cells between engrafter and age-matched controls. (C) The selection ratio for donor-specific Ly49D+ NK cell phenotypes (%Ly49D+AFG+/%Ly49D+AFG2) in engrafter mice was several times that observed in control mice, whereas the selection ratio for nonspecific Ly49D+ NK cell phenotypes (%Ly49D+AFG+/%Ly49D+AFG2) was similar to controls. (D) The overall frequency of NK cell bone marrow spleen and peripheral blood is reduced in engrafter chimeras compared with age-matched controls. Subset analysis reveals that the reduction in frequency of NK cells is proportionate to the loss of hostile NK cells in the bone marrow, spleen, and peripheral blood. A CD3+ NK1.1 lymphocyte gate was used to define the total NK cell pool. Data represent mean frequency ± SD for at least five separate animals in either group. *p < 0.05.

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Alternatively, in the absence of a strong inhibitory signal, hostile NK cells may have persisted in the engrafter mice in a hyporesponsive state, as has been shown for NK cells lacking self-specific inhibitory receptors in naive B6 mice (27–29). In support of this hypothesis, hostile NK cells from engrafter mice were markedly hyporesponsive relative to friendly NK cells following stimulation with plate-bound Ly49D or NK1.1 mAb (Fig. 4A) using an established in vitro assay for NK cell responsiveness (30). This was in sharp contrast to hostile NK cells in naive control mice, which were slightly more responsive than friendly NK cells in the same assays (Fig. 4A). Additionally, the response to calcium flux (PMA and ionomycin) was maintained, confirming that the hostile NK cells retained the capacity to produce IFN-γ.

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of the more mature R2 and R3 phenotypes in the bone marrow. A similar pattern for R1 and R2 persisted in the spleen and blood, where the NK cell repertoire is progressively dominated by the more mature R2 and R3 subset. The differences were not significant in the lung, where relatively few immature NK cells are typically recovered. Conversely, friendly NK cells displayed no significant changes in maturation when compared with controls.

To determine whether a diminished avidity for the donor cells contributed to tolerance, the cell surface expression of Ly49D by hostile NK cells was examined in engrafted mice. Fig. 5C illustrates that hostile Ly49D+AFG⁺ NK cells in engrafted mice expressed significantly lower levels of Ly49D on their cell surface when compared with controls, whereas a reduction in Ly49D expression was not exhibited by friendly NK cells. The decreased expression appeared to be specific for Ly49D, as the expression level of Ly49H was unchanged when coexpressed on the same hostile NK cells (Fig. 5C). Furthermore, Ly49D downregulation was not observed with coexpression of a donor-specific inhibitory receptor in the Ly49D⁺AFG⁻ NK cell subsets in engrafted mice (Fig. 5C). A kinetic analysis revealed that the Ly49D downregulation was clearly evident by 3 wk of age and was maintained throughout the period of Ly49 receptor maturation (Fig. 5D). These selective changes in the level of Ly49D expression offer an explanation for the anergic response of hostile NK cells to anti-Ly49D stimulation. However, the same hostile NK cells were also hyporesponsive to anti-NK1.1 stimulation without changes in the cell surface expression of NK1.1 (Supplemental Fig. 3), raising the possibility that the cell-intrinsic responses to activating receptor signaling were fundamentally altered in hostile NK cells. Further study is needed to confirm whether the impairment in multiple signaling mechanisms arises independently or through a common defect in a downstream signaling pathway.

Despite widely disparate frequencies, friendly and hostile NK cells from engrafted mice exhibit equivalent rates of homeostatic proliferation

Previous studies of NK cell maturation reveal a higher rate of homeostatic proliferation by NK cells that express a self-specific inhibitory receptor compared with NK cells that do not (23). Accepting this principle, we would expect that the friendly NK cells in engrafted mice would display a higher homeostatic proliferation rate compared with the hostile NK cells. To evaluate this possibility, an in vivo BrdU assay was performed at different phases of murine NK cell development (24). A phenotypic analysis of friendly and hostile NK cells in the spleens of engrafted mice (Fig. 6A) revealed a skewed selection ratio that mirrored the peripheral blood findings (Fig. 3C) beginning at the earliest stages of Ly49 receptor expression and reaching a plateau as receptor expression matured. Despite the markedly diverging frequencies of these alternate phenotypes in the engrafted mice, the homeostatic proliferation of friendly and hostile NK cells was nearly identical,yielding a proliferation ratio that was approximately equal to 1 (Fig. 6B). As a result, the preponderance of friendly NK cells could not be explained by differences in homeostatic proliferation.

FIGURE 4. Hostile NK cells from engrafted mice are selectively anergic to activating receptor-mediated signaling. Splenocytes from engrafters and controls were subjected to in vitro stimulation by plate-bound anti-Ly49D (4E5) mAb, anti-NK1.1 (PK136) mAb, or PMA and ionomycin, and the frequency of IFN-γ⁺ cells was measured. (A) The resulting hostile:friendly IFN-γ production ratio was calculated and compared between the groups: %Ly49D⁺AFG⁺ IFN-γ⁺ NK cells/ %Ly49D⁺AFG⁺ IFN-γ⁺ NK cells. (B) In separate experiments, a subset analysis of Ly49D⁺ NK cells coexpressing single or multiple donor-specific inhibitory receptors was performed. The IFN-γ production ratio for each Ly49D⁺ NK cell subset relative to the friendly NK cell subset was calculated and compared: %subset⁺ IFN-γ⁺ cells/ %Ly49D⁺AFG⁺ IFN-γ⁺ NK cells. Values shown are derived from analysis of gated CD3⁻NK1.1⁺ lymphocytes with five separate animals in either group plus or minus SD. Values for Ly49D⁺AFG⁺ NK cells are excluded, as the frequency of this subset is <1% of all Ly49D⁺ NK cells and inadequate for statistical analysis. *p < 0.05.
Hostile NK cells in developmentally immature prenatal chimeras upregulate inhibitory receptors through activating receptor recognition of cognate donor ligand

The higher selection ratio in engraver mice might be alternatively explained by the upregulation of inhibitory receptor expression by hostile NK cells in engraver mice, resulting in the peripheral crossover to a friendly phenotype. To examine this possibility, hostile NK cells were sorted from naive B6Ly5.1 control mice and adoptively transferred to BALB/c → B6Ly5.2 engraver mice or B6Ly5.2 controls (Fig. 7A). The donor mice were either immature (3 wk) or mature (7 wk) with regard to Ly49 receptor acquisition. The upregulation of inhibitory receptor expression by the transferred hostile Ly5.1+ NK cells was tracked in the new chimeric or nonchimeric (naive) secondary environment. As shown in Fig. 7B, hostile NK cells that were transferred from an immature donor to a chimeric secondary recipient displayed a sharp upregulation in the expression of the donor-specific inhibitory receptors, eventually reaching the same relative frequency of friendly NK cells seen in mature engraver mice. The secondary recipient mice did not display any signs of autoimmunity, and their chimerism level was stable throughout the study period despite the transfer of these cells (data not shown). Conversely, in the nonchimeric control environment, the transferred hostile NK cells exhibited much less inhibitory receptor upregulation and only reached the level seen in naive mice. Surprisingly, hostile NK cells from mature donor mice did not have the same capacity for inhibitory receptor upregulation as those from immature donors (Fig. 7C). Lastly, adoptively transferred Ly49D AFG- NK cells exhibited a stable phenotype without upregulation of Ly49D, Ly49A, Ly49F, or Ly49G in the chimeric secondary host, suggesting that signaling input from the activating receptor is needed for inhibitory receptor upregulation (Supplemental Fig. 4). Therefore, the expression of donor-specific inhibitory receptors by hostile NK cells is favored by activating receptor recognition of donor ligand in the peripheral environment and most likely contributes to the preponderance of phenotypically friendly NK cells in engraver mice. The capacity for these adaptive changes appeared to be lost in developmentally mature mice.

**FIGURE 5.** Nondeleted hostile NK cells in stable chimeras exhibit an immature low-avidity phenotype. (A) Multiorgan analysis at 12 wk of age, demonstrating the relative maturation of Ly49D, hostile and friendly NK cells in an engraver and a control mouse using the CD27/CD11b paradigm: CD27<sup>high</sup>/CD11<sup>low</sup> (R1); CD27<sup>high</sup>/CD11<sup>high</sup> (R2); CD27<sup>low</sup>/CD11<sup>high</sup> (R3). (B) Analysis of hostile and friendly NK cell maturation at 5 mo of age revealing the selectively impaired maturation of hostile NK cells in the bone marrow, spleen, and blood of engraver mice. (C) Measurement of the relative intensity of Ly49D expression by splenic NK cells from engraver and control mice at 12 wk of age reveals selective downregulation by hostile phenotypes. The coexpression of a single donor-specific inhibitory receptor is sufficient to prevent Ly49D downregulation. The graphed data represent the mean fluorescence intensity of Ly49D receptor expression by friendly NK cells from at least five engraver mice relative to the corresponding levels exhibited by control mice (mMFI). (D) Ly49D expression was tracked on friendly and hostile peripheral blood NK cells from engraver and naive control mice from 3 to 12 wk of age. A CD3-NK1.1 lymphocyte gate was used to define the total NK cell pool. Values shown are derived from at least five separate animals in either group ± SD. *p < 0.05.
Discussion

From the results of this study, a series of novel principles emerge that may fundamentally guide future investigations of developmental NK cell allospecific education. The first of these principles is that host NK cells expressing a donor-reactive activating receptor are key effectors of the prenatal allospecific response. This finding establishes a mechanistic basis for an immune barrier to IUHCT that resides within the host. In prenatal BALB/c→B6 murine chimeras, this immune barrier exists as a subset of Ly49D+ NK cells. In the human fetus, it may exist in subsets of NK cells expressing activating killer Ig-related receptors (35). If the principle is well conserved, then the potential for an allospecific immune response might include NK cells expressing killer Ig-related receptors for ligands that do not normally exist within the host. Surprisingly, the short-term depletion of the Ly49D+ NK cell subset reliably preserved long-term engraftment in a subset of the rejecter mice even after the treatment was discontinued. The potential to rescue engraftment in this way suggests that the targeted depletion of donor-reactive NK cells may be a useful adjunct for clinical IUHCT.

The second principle is that the appraisal for self-reactivity during NK cell selection in prenatal chimeras is based on the composite receptor phenotype and is directed by activating receptor recognition of the donor ligands. This concept was first examined by George et al. (36), who found that 97.8% of Ly49D+ NK cells in B6×Balb F1 hybrid mice (H2d/s) coexpressed at least one H-2Dd-specific inhibitory receptor and concluded that this was necessary to convey tolerance to the Ly49D+ NK cells. This conclusion is advanced by the findings in prenatal chimeras that support phenotypic selection as the primary tolerance mechanism functioning at the earliest stages of Ly49 receptor development. Activating receptor recognition of the donor ligands appeared to regulate the selection process because the Ly49D2 NK cells displayed no differences in inhibitory receptor expression between the engrafter and control mice. This stark contrast between Ly49D+ and Ly49D2 NK cells indicates that the checkpoint for self-reactivity occurs after the expression of the Ly49D+ receptor with the potential to subsequently coexpress Ly49A, Ly49F, or Ly49G.

A third principle is that central NK cell selection is supported by the peripheral upregulation of inhibitory receptor expression by hostile NK cells following recognition of the donor ligands through the activating receptor. Whether this mechanism is a primary feature of NK cell education or a secondary adaptive process was addressed in the current report. The observation that the capacity for inhibitory receptor upregulation is greatly diminished in developmentally mature mice suggests that this is the primary process through which the final NK cell repertoire is attained. However, although a mechanism involving inhibitory receptor upregulation might explain the increased frequency of friendly NK cells, it does not account for the overall decrease in the total number of NK cells seen in the engrafter mice. Remarkably, we found very few hostile NK cells in the engrafter mice at any time point even as early as 2 wk of age (Fig. 6A), suggesting that the elimination of this
phenotype occurred at a very early stage in NK cell maturation. The similarities in homeostatic proliferation between hostile and friendly NK cells at early and late time points (Fig. 6B) further support that tolerance was established at a very early age. Hence, a model of NK cell education that begins with phenotypic selection in the bone marrow and is subsequently supported by a developmentally limited capacity for fine-tuning of the peripheral repertoire provides a more comprehensive explanation for these findings. Instructive recognition of ligand through the activating receptor appears to be an essential feature of each step. Conclusive support for such a process awaits the future characterization of stage-specific markers for NK cell education.

A fourth principle is that the terminal development of hostile NK cells is fundamentally altered in engrafted mice and results in a state of selective anergy. The pattern of altered activating receptor expression and maturation displayed by NK cells from the bone marrow, spleen, and peripheral blood further suggests that the appraisal for self-responsiveness began during the final stages of NK cell maturation in the bone marrow. Low-avidity, immature hostile NK cells may have exited the selection process and relocalized to peripheral sites such as the spleen, where they remained relatively hyporesponsive to receptor-mediated signaling. Thereafter, defective signaling through the activating receptor may have been insufficient to trigger upregulation of the inhibitory receptors in these cells. These alterations clearly result following development in the chimeric environment.

A number of previous reports have advanced a disarming model for developmental regulation of potentially self-reactive NK cells and are germane to the analysis of the current findings (37). Accordingly, hostile Ly49D+AFG+ NK cells in BALB/c→B6 prenatal chimeras must be disarmed due to their unrestrained reactivity toward the donor. This could be brought about by the unopposed chronic Ly49D stimulation of hostile NK cells resulting in the exhaustion of DAP10 and DAPI2 signaling adaptors leading to the functional hyporesponsiveness of other DAP10/12-dependent activating receptors and potentially DAP10/12-independent receptors through an additional downstream signaling defect (38–40). In this way, disarming would explain the pattern of anergy exhibited by the hostile NK cells. However, disarming does not provide a stand-alone explanation for the emergence of only a small percentage of Ly49D+AFG+ NK cells at the earliest developmental time points. Instead, disarming may serve as the prevailing adaptive process in developmentally mature mice rather than during NK cell education in immature mice, where the capacity for inhibitory receptor upregulation is preserved (Fig. 7C). If the capacity to induce the coexpression of ligand-specific inhibitory receptors during development is not possible, broad disarming of NK cells expressing the activating receptor may occur. Relevant to this postulate, Tripathy et al. (12–14) examined the effect of developmental exposure of the m157 viral ligand to its exclusive DAP10/12-dependent activating receptor in transgenic mice in which no known m157-specific inhibitory receptor has been defined. The authors observed hyporesponsiveness in Ly49H+ NK cells to DAP10/12-dependent and independent activation without alteration in the expression of inhibitory Ly49 receptors. In addition to the lack of a known m157-specific inhibitory receptor, differences in ligand-binding affinity, stromal interaction, cis versus trans recognition, membrane confinement, and synapse formation are all possible determinants for the manner in which the activating receptor input was managed differently during NK cell education between the m157 transgenic mice and the prenatal.

**Figure 7.** Adoptive transfer of hostile NK cells into a chimeric environment results in upregulation of inhibitory receptor coexpression. (A) Schematic representation of the adoptive transfer experiments. Hostile NK cells from either immature (3 wk) or mature (7 wk) naive B6Ly5.1 mice were harvested and sorted to isolate hostile NK cells. The hostile NK cells were adoptively transferred to mature engrafters and age-matched B6Ly5.2 controls. The adoptively transferred cells were followed for their expression of the donor-specific inhibitory receptors. (B) The upregulation of inhibitory receptors in the adoptively transferred hostile NK cells from both the immature and mature donor is plotted. This is reported as the fraction of Ly5.1+ NK cells that are Ly49D+AFG+ and is followed serially in peripheral blood. (C) The upregulation of inhibitory receptor is compared between mature and immature donor NK cells transferred into the chimeric environment. A CD3+ NK1.1 lymphocyte gate was used to define the total NK cell pool. Data are derived from at least three separate experiments in each group ± SD. *p < 0.05.
chimera models (41–44). As such, the developmental regulation of hostile NK cells in prenatal chimeras was more complex than could be predicted by current models. The present study adds the conclusion that instructive recognition through the activating receptor during development can regulate the coexpression of ligand-specific inhibitory receptors, or alternatively, the aborted maturation of low-affinity Ly49D<sup>+</sup> NK cells that do not coexpress a relevant inhibitory receptor. A better understanding of the mechanistic regulation of these alternate fates awaits stage-specific characterization of developing hostile NK cells.

A final principle suggested by the current findings pertains to the role of the donor-specific activating receptor in the licensing of developing NK cells. Proposed mechanisms of NK cell licensing account for the preserved frequency and functionality of Ly49D-mediated activation in friendly Ly49<sup>D<sub>A</sub></sup> phenotype NK cells following the trans recognition of the donor H-2<b>D</b> ligand (28, 43, 44). However, current models of NK cell licensing do not explain the absence of enhanced functionality of Ly49D<sup>A</sup> over Ly49D<sup>F</sup> AFG<sup>+</sup> cells in BALB/c→B6 prenatal chimeras. This departure suggests a role for the Ly49D<sup>+</sup> activating receptor in the licensing of NK cells in prenatal chimeras and warrants further examination. In support of this possibility, a recent study by Wickstro¨m et al. (45) in a CD1-defective strain found an unexpected loss of the pattern of NK cell licensing and of inhibitory receptor coexpression and warrants further examination. The authors have no financial conflicts of interest.

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Disclosures
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References
Supplemental Figure 1

Efficacy of in vivo Ly49D depletion. To determine the effectiveness of in vivo depletion of Ly49D+ NK cells, control mice received a single IP injection of anti-Ly49D (4e5) mAb and were sacrificed several days later for multi-organ analysis by flow cytometry. (A) The frequency of Ly49D+CD3-NK1.1+ lymphocytes was measured using an alternative staining approach with the YE148 (Ly49A) and 12A8 (Ly49A/D) mAb’s to avoid competition with the 4E5 mAb used for depletion. (B) Sample histograms showing the frequency of Ly49D single positive NK cells (12A8+YE148-) in control or 4E5-depleted mice are shown for the various hematopoietic organs analyzed in a single mouse. These results for the 4E5-depleted group are representative of 3 separate mice receiving identical treatment.

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Supplemental Figure 2

Co-expression of Ly49C (4LO-3311), Ly49I (YLI-90) or NKG2A (16A-11) by friendly and hostile NK cells is similar between control and engrafter mice. The co-expression of any combination of Ly49C, Ly49I, or NKG2A (CIN+) or the absence of any Ly49C, Ly49I, or NKG2A co-expression (CIN-) by (A) all NK cells, (B) Ly49D+ NK cells, (C) Ly49D- NK cells, (D) friendly NK cells or (E) hostile NK cells was determined in the peripheral blood of control and engrafter mice. Values shown are derived from at least 5 separate animals in either group ± SD and were compared using a two-tailed Student t test assuming unequal variances (p=NS).
Supplemental Figure 3

NK1.1 expression by friendly and hostile NK cells. Cell surface NK1.1 expression of engrafter mice relative to levels on control mice (rMFI) was tracked on friendly and hostile peripheral blood NK cells from 3 – 12 weeks of age. Values shown are derived from at least five animals in either group ± SD, (p=NS).
Supplemental Figure 4
Adoptive transfer of naive Ly49D-AFG- NK cells to chimeric secondary environment. Ly49D-AFG-NK cells from immature (3 weeks) naïve B6Ly5.1 mice were harvested and sorted from pooled splenocytes. The Ly49D-AFG- NK cells were then adoptively transferred to a mature B6Ly5.2 engrafted (7 weeks) and followed serially in PB for their expression of the donor-specific inhibitory receptors. The upregulation of inhibitory receptors in the adoptively transferred Ly49D-AFG- NK cells is plotted as the fraction of Ly5.1+ NK cells that are Ly49D-AFG+. No Ly49D+ NK cells of donor origin were detected and therefore do not appear in the graph.