The Innate NK Cells, Allograft Rejection, and a Key Role for IL-15

Alexander Kroemer, Xiang Xiao, Nicolas Degauque, Karoline Edtinger, Haiming Wei, Gulcin Demirci and Xian Chang Li

*J Immunol* 2008; 180:7818-7826; doi: 10.4049/jimmunol.180.12.7818

http://www.jimmunol.org/content/180/12/7818
The Innate NK Cells, Allograft Rejection, and a Key Role for IL-15

Alexander Kroemer,* Xiang Xiao,* Nicolas Degauque,* Karoline Edtinger,* Haiming Wei, † Gulcin Demirci,* and Xian Chang Li2*†

Transplant rejection is mediated primarily by adaptive immune cells such as T cells and B cells. The T and B cells are also responsible for the specificity and memory of the rejection response. However, destruction of allografts involves many other cell types including cells in the innate immune system. As the innate immune cells do not express germline-encoded cell surface receptors that directly recognize foreign Ags, these cells are thought to be recruited by T cells to participate in the rejection response. In this study, we examined the alloreactivity of the innate NK cells in Rag−/− mice using a stringent skin transplant model and found that NK cells at a resting state readily reject allogeneic cells, but not the skin allografts. We also found that IL-15, when preconjugated to its high affinity IL-15Rα-chain, is remarkably potent in stimulating NK cells in vivo, and NK cells stimulated by IL-15 express an activated phenotype and are surprisingly potent in mediating acute skin allograft rejection in the absence of any adaptive immune cells. Furthermore, NK cell-mediated graft rejection does not show features of memory responses. Our data demonstrate that NK cells are potent alloreactive cells when fully activated and differentiated under certain conditions. This finding may have important clinical implications in models of transplantation and autoimmunity. The Journal of Immunology, 2008, 180: 7818–7826.

Transplant rejection is traditionally believed to be a T cell-mediated event (1, 2). However, the daunting difficulty in creating transplant tolerance by using T cell-centric protocols under stringent conditions stimulates renewed interests in the role of innate immune cells, especially NK cells, in transplant rejection (3, 4). In fact, NK cells are a major cell type in the innate immune system and constitute the third largest population of lymphocytes besides T cells and B cells in the periphery (5). Unlike T cells and B cells, the innate NK cells are developmentally programmed to kill targets without prior Ag priming (5, 6). It is well known that NK cells in the periphery express both clonotypic stimulatory and inhibitory receptors that are specific for self MHC class I (MHC I)3 molecules (6), and engagement of the inhibitory receptors by self MHC I molecules is the principal mechanism by which NK-mediated damage to self is prevented (6–8). Thus, in transplant models in which donors and recipients are mismatched for the MHC I molecules, alloreactive NK cells directed against foreign targets are readily induced (9). Indeed, in bone marrow transplant models, NK cells are potent effector cells in rejection of allogeneic stem cells (10, 11). In solid organ transplant models, however, the role of NK cells remains enigmatic. NK cells are frequently found in large numbers in rejecting allografts (3, 12), but NK cells by themselves are neither necessary nor sufficient for complete rejection of solid organ transplants (13). The lack of graft rejection by NK cells is not due to their functional impairment, because NK cells can effectively kill graft-derived allogeneic APCs, which in return promote engraftment (13). It is not clear why NK cells, which readily reject allogeneic cells, fail to reject solid allografts from the same donors.

Besides being regulated by cell surface receptors that can deliver either stimulatory or inhibitory signals (6), NK cells are also highly responsive to the common γc cytokines, especially IL-2 and IL-15 (14). In fact, IL-15 plays a particularly important role in development and survival of NK cells (15). It is not clear how the cytokine-mediated signals interact with signals from the NK cell surface receptors in dictating the functional status of NK cells, but several recent studies suggest that NK cells in the periphery may require an additional maturation process to gain full functional competence (16, 17), suggesting a possibility that the effects of NK cells in the allograft response may be regulated by differences in their activation status. In the present study, we took an in vivo approach to critically examine the alloreactivity of NK cells in mediating skin allograft rejection. We found that the innate NK cells in Rag−/− mice can mediate prompt skin allograft rejection independent of any adaptive immune cells, but this effect requires activation and further effector differentiation of NK cells in vivo. Importantly, IL-15 is of central importance in activating NK cells in the rejection response.

Materials and Methods

Animals

DBA/2 (H-2d), C57BL/6 (H-2b), and Rag−/− (H-2b) mice were purchased from The Jackson Laboratory. Rag−/−γc−/− double mutant mice (H-2b) were obtained from the Taconic Farms. Animal care and use conformed to the guidelines established by the Animal Care Committee at Harvard Medical School in Boston, MA.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
Reagents

The following anti-mouse mAbs were purchased from eBioscience, BD Biosciences, and BioLegend: Alexa Fluor 488-anti-mouse NK1.1, PE- and PE-Cy7 anti-mouse NK1.1 (clone PK136), FITC-anti-CD49b, PE-anti-CD49b (clone DX5), PE-anti-CD122 (clone TM-b1), PE-anti-NK2GD (clone CX5), PE-anti-CD44 (clone IM7), PE-anti-CD62L (clone MEL-14), PE-anti-B220 (clone RA3-6B2), FITC-anti-mouse 2B4 (clone 2B4), PE-anti-CD16 (clone 93), PE-anti-Granzyme B (clone CX5), PE-anti-CD62L (clone MEL-14), and isotype control Abs.

Soluble IL-15/IL-15R complex preparation and in vivo injection

Human recombinant IL-15 was purchased from R&D Systems. A recombinant fusion protein consisting of the ectodomain of the mouse IL-15a-chain and the human IgG1 Fc (IL-15Rα/Fc) was also purchased from R&D Systems. The IL-15/IL-15Rα/Fc complex was prepared as previously reported (18). In brief, both IL-15 and IL-15Rα/Fc were resuspended in PBS solution and then mixed together at a ratio of 1:6 (IL-15 to IL-15Rα/Fc) in terms of protein mass. The mixture was incubated at 37°C for 30 min and then used for in vivo injection. For stimulation of NK cells in vivo in Rag2−/− mice, each mouse was given 2.5 μg of IL-15/IL-15Rα/Fc complex i.p. in 200 μl of PBS, and all analyses of NK expansion were performed 4 days later in the treated mice. In some experiments, Rag2−/−γc−/− mice treated with the same IL-15 protocol were included as controls.

Cell staining and flow cytometry

Cells in the spleen and lymph nodes as well as in the extra-lymphoid organs (e.g., liver and lungs) were prepared from donor mice. Cells were resuspended in PBS/0.5% BSA at 1 × 10^6 cells/ml and stained with fluorochrome-conjugated Abs on ice for 20 min. After the staining, cells were washed twice in PBS/BSA and fixed in 1% paraformaldehyde before FACS analysis. For intracellular granzyme B staining, cells were first fixed and cell membrane permeabilized in PermFix solution, followed by staining with PE-anti-mouse granzyme B (clone 16G6) mAb. Samples were acquired using the FACSScan or LSR II flow cytometer (BD Biosciences), and data analysis was performed using the FlowJo software (Tree Star).

In vivo cytotoxicity assay

Splenic cells were prepared from donor C57BL/6 and DBA2 mice, and cells were labeled with 0.1 and 2.5 μM CFSE, respectively (19). The CFSE-labeled B6 and DBA2 cells were mixed together at 1:1 ratio, and the cell mixture was injected into Rag2−/− and Rag2−/−γc−/− mice via the tail vein. Each mouse was given 10×10^6 cells. The host mice were killed 20 h later and survival of CFSE-labeled donor cells in the host spleen was analyzed using a FACScan flow cytometer.

In vivo BrdU uptake assay

Rag2−/− mice were injected i.p. with 1.5 mg of BrdU (BD Biosciences) with or without treatment with IL-15/IL-15R complex. Splenic cells were prepared from BrdU-injected mice and non-BrdU-treated control mice 12 h later. Cells were stained for NK1.1 plus DX5, and then subsequently stained for intracellular BrdU (BrdU Flow Kit; BD Biosciences) according to the manufacturer’s instructions. Incorporation of BrdU in NK cells was determined using a LSR II cytometer.

Cell sorting

Splenic cells were prepared from Rag2−/− mice with or without IL-15/IL-15R treatment and briefly stained with FITC-anti-DX5 and PE-Cy7-anti-NK1.1. The NK1.1+ DX5+ NK cells were identified, electronically gated, and then selectively sorted using the MoFlo high-speed cell sorter (Dako Cytomation). The purity of cells sorted using this method is consistently >99% (20). The FACS-sorted NK cells were used for the real-time PCR analyses.

Real-time PCR

Real-time RT-PCR was performed as previously reported (21). In brief, total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA with ABI Prism TaqMan reverse transcription method. Expression of genes of interest and of GAPDH control was calculated according to the 2^−ΔΔCt formula as provided by the manufacturer (ABI PRISM 7700 user bulletin, Applied BioSystems) and expressed as an arbitrary unit.

FIGURE 1. NK cells readily reject allogeneic cells but not allogeneic skin grafts. a, Splenic cells were prepared from Rag2−/− and Rag2−/−γc−/− mice and stained with anti-NK1.1 mAb to examine the presence of NK cells. Data from four individual experiments are shown. b, CFSE-labeled B6 (CFSEhi) and DBA2 (CFSElo) spleen cells were mixed at a 1:1 ratio and then injected into Rag2−/− and Rag2−/−γc−/− hosts. Cells were recovered from the host spleen 20 h later; the ratio of CFSE-labeled cells was determined by FACS and shown in relative percentage. Data are representative of three independent experiments. c, DBA2 skin allografts (H-2b) were transplanted onto Rag2−/− and Rag2−/−γc−/− mice (H-2b) and the skin allograft survival was determined and plotted in a Kaplan-Meier plot. d, DBA2 skin allografts transplanted onto Rag2−/− and Rag2−/−γc−/− mice were harvested 7 days after engraftment. Total cellular RNA was extracted from the skin allografts and then reverse transcribed into cDNA. NK1.1 gene transcripts were quantified by real-time PCR. Data shown are representative of three independent experiments.

a: p < 0.05.
**Histopathology**

Donor skin grafts were excised from recipient mice, fixed in 10% formalin, and embedded in paraffin. Serial tissue sections (5 µm) were cut and mounted on slides (Fisher Scientific), fixed in methanol, and stained in H&E for identification of tissue damage and cellular infiltrates. Images were captured using a Nikon Eclipse 80i system. For immunofluorescence staining, tissue samples were frozen in liquid nitrogen and isopentane, serial tissue sections (5 µm) were fixed in 4°C acetone for 10 min. After initial blocking with serum, sections were incubated overnight at 4°C with Alexa Fluor 488-anti-NK1.1 Ab (BioLegend, 1/100 dilution). Cell nuclei were counterstained blue with Hoechst 33258. Images were captured using a Zeiss ApoTome system.

**Skin transplantation**

Full thickness body skin grafts (~1 cm²) from donors (H-2d) were transplanted onto the thoracic wall of recipient mice (H-2b). The skin graft was secured with an adhesive bandage for the initial 5 days. Graft survival was

![Image](http://www.jimmunol.org/...)

**FIGURE 2.** Massive expansion of NK cells in vivo after treatment with IL-15/IL-15Rα complex. 

- **a**, Rag-/- mice were injected i.p. with 1.5 mg BrdU 1 day after treatment with IL-15/IL-15Rα complex. Spleen cells were prepared 12 h later, and cells were permeabilized and stained with FITC-anti-BrdU mAb. BrdU uptakes in the NK1.1+ fraction were presented. Numbers indicate the frequencies of BrdU+ cells among total NK1.1+ spleen cells. Data shown are representative of three independent experiments. 
- **b**, Rag-/- mice were treated with IL-15/IL-15Rα complex, NK1.1+ DX5+ NK cells were FACS sorted from the treated mice 4 days later, and the total cellular RNA was extracted from the NK cells. Expression of Bcl-2, Bcl-xL, and survivin transcripts was quantified by real-time PCR. Data shown are representative of three independent experiments. 
- **c** and **d**, Rag-/-/γc-/- mice were treated with IL-15/IL-15Rα complex or a control Ig. Four days later, cells from spleen and blood were labeled with FITC-anti-NK1.1 and PE-anti-DX5 mAbs. NK cells expressing both NK1.1 and DX5 were analyzed by FACS and compared. The absolute cell number was calculated based on the total numbers of cells recovered and presented as mean ± SD of four animals in each group. Rag-/-/γc-/- mice treated with the same IL-15 protocol were included as controls. The data are representative for at least four independent experiments. *p < 0.05.
then followed by daily visual inspection. Rejection was defined as a loss of >80% of the transplanted skin tissue (13).

Statistics
Allograft survival was compared using the log-rank test. Other data were compared using a Student t test. A p < 0.05 was considered as significant.

Results
NK cells, while cytolytic to allogeneic cells, fail to reject skin allografts
To address the issue of NK alloreactivity in transplant models, we used Rag−/− and Rag plus the common γ-chain double deficient mice (Rag−/−γc−/−) (H-2b) as transplant recipients and studied skin allograft rejection by such recipient mice. Both Rag−/− and Rag−/−γc−/− mice lack T and B cells. Unlike the Rag−/−γc−/− mice, which are also deficient for NK cells (22), the Rag−/− mice have a large population of NK cells in the periphery (Fig. 1a) (23–25). Thus, the role of NK cells in mediating the skin allograft rejection can be precisely studied in the complete absence of any adaptive immune cells. To ascertain the cytolytic activity of NK cells in Rag−/− mice, we adoptively transferred CFSE-labeled

syngeneic (C57BL/6, H-2b) and allogeneic (DBA/2, H-2b) spleen cells (mixed at 1:1 ratio) into Rag−/− and Rag−/−γc−/− hosts (H-2b), and cell survival was examined 20 h later. As shown in Fig. 1b, both CFSE-labeled B6 and DBA/2 cells could be readily recovered at equal numbers from Rag−/−γc−/− hosts, suggesting that both cell types survive equally well in the Rag−/−γc−/− mice. In the Rag−/− mice, however, the allogeneic DBA/2 cells, but not syngeneic B6 cells, were promptly rejected, suggesting a strong NK-mediated rejection of allogeneic cells in the Rag−/− mice. We then transplanted DBA/2 skin allografts onto Rag−/− and Rag−/−γc−/− mice and examined the skin allograft survival. Consistent with our previous report (13), the skin allografts survived indefinitely in both Rag−/− and Rag−/−γc−/− mice (>100 days) without any signs of rejection (Fig. 1c). Analysis of DBA2 skin allografts transplanted onto Rag−/− and Rag−/−γc−/− mice by real-time RT-PCR demonstrated copious amounts of NK1.1 gene transcripts in the Rag−/−, but not in the Rag−/−γc−/− recipients (Fig. 1d), suggesting that the skin allografts are likely infiltrated by host NK cells despite long-term survival in Rag−/− mice. Thus, despite strong cytolytic activities against allogeneic DBA2 cells, NK cells by themselves fail to reject the DBA2 skin allografts.

FIGURE 3. Activation and functional maturation of NK cells in IL-15/IL-15Ra complex treated mice. a, Rag−/− mice were treated with IL-15/IL-15Ra complex or a control Ig. Splenic cells from the treated animals were prepared 4 days later and labeled with FITC-anti-NK1.1 and PE-Cy7-anti-DX5 mAbs to select NK1.1+DX5+ NK cells. Expression of other cell surface markers associated with NK activation by the NK1.1+ fraction was analyzed by FACS. Data shown are representative of three to five individual experiments. b, Rag−/− mice were treated with IL-15/IL-15Ra complex or a control Ig. Splenic cells from the treated animals were prepared 4 days later and labeled with FITC-anti-NK1.1 and PE-Cy7-anti-DX5 mAbs. NK1.1+DX5+ splenic NK cells were FACS sorted, and expression of gene transcripts for effector molecules, cytokines, chemokines, and chemokine receptors was quantified by real-time PCR. Data shown are representative of three independent experiments.
IL-15 stimulates expansion and functional maturation of NK cells in vivo

To test the hypothesis that NK-mediated rejection depends on the activation status of NK cells, we treated Rag<sup>−/−</sup> mice with an IL-15/IL-15R<sub>a</sub> complex to maximally stimulate NK cells in vivo. This complex was chosen because IL-15 is a product of active immune cell activation (26). In addition, the IL-15/IL-15R<sub>a</sub> complex has demonstrated efficacy in activating NK cells in vivo (18, 27).

As shown in Fig. 2a, treatment of Rag<sup>−/−</sup> mice with IL-15/IL-15R<sub>a</sub> complex vigorously stimulated proliferation of NK cells as determined by BrdU uptake assays. Furthermore, NK cells markedly up-regulated the expression of cell survival genes including Bcl-2, Bcl-xL, and survivin after IL-15 stimulation (Fig. 2b), consistent with the notion that IL-15 is important in survival of NK cells (28). Flow cytometry analysis demonstrated a massive expansion of NK cells in IL-15-treated Rag<sup>−/−</sup> mice, but not in IL-15-treated Rag<sup>−/−</sup>γ<sup>−/−</sup> mice. As compared with the control Ig-treated mice, the absolute number of NK cells in the spleen and blood of IL-15 treated mice was increased by greater than 10-fold after IL-15 treatment (Fig. 2, c and d). Phenotypically, the IL-15-expanded NK cells up-regulated the cell surface expression of CD44, 2B4, NKG2D, CD16, and B220, suggesting an activated phenotype (29). It is noteworthy that expression of the pan-NK markers NK1.1 and DX5 was uniform on the IL-15-stimulated NK cells as compared with the controls (Fig. 3a), indicating that IL-15 stimulates all NK cells. Importantly, NK cells from IL-15-treated mice had increased expression of effector molecules and certain chemokine and chemokine receptors as compared with the controls (Fig. 3b). The up-regulation of perforin and granzyme B is particularly impressive, with several hundred-fold higher than that in resting NK cells (Fig. 3b). Similar changes were observed in NK cells isolated from bone marrow, peripheral blood, liver, and lungs (data not shown). These results suggest that NK cells can acquire an activated phenotype and undergo further effector maturation after IL-15 stimulation in vivo.

NK cells expanded by IL-15 in vivo mediate prompt skin allograft rejection in the absence of any adaptive immune cells

To determine the alloreactivity of activated NK cells in transplant models, we again transplanted the DBA/2 skin allografts onto Rag<sup>−/−</sup> and Rag<sup>−/−</sup>γ<sup>−/−</sup> mice treated with or without IL-15/IL-15R<sub>a</sub> complex and the skin allograft survival was examined. As expected, the untreated Rag<sup>−/−</sup> and Rag<sup>−/−</sup>γ<sup>−/−</sup> mice accepted the DBA/2 skin allografts long-term (Fig. 4a). IL-15-treated Rag<sup>−/−</sup> and Rag<sup>−/−</sup>γ<sup>−/−</sup> mice, which lack NK cells, also accepted the DBA/2 skin allografts indefinitely (Fig. 4a). To our surprise, treatment of Rag<sup>−/−</sup> mice with the IL-15/IL-15R<sub>a</sub> complex induced acute skin allograft rejection, and 7 out of 11 (64%) IL-15-treated Rag<sup>−/−</sup> recipients completely rejected the DBA/2 skin allografts within 18 days after transplantation (Fig. 4a). Interestingly, 4 out of 11 (36%) treated Rag<sup>−/−</sup> mice showed signs of acute rejection crisis early after transplantation, with ~50% of the transplanted skin tissue being rejected, but the remaining half of the grafts survived long-term (Fig. 4b). This finding suggests that rejection did occur in all IL-15-treated Rag<sup>−/−</sup> mice, but some recipient mice failed to completely eliminate the skin allografts despite an early rejection crisis. Rejection is not due to the side effects of the IL-15/IL-15R<sub>a</sub> complex on the skin grafts as Rag<sup>−/−</sup> mice transplanted with syngeneic skin graft and treated with the IL-15/IL-15R<sub>a</sub> complex accepted the skin grafts (Fig. 4a). Histologically, graft rejection by IL-15 treated Rag<sup>−/−</sup> mice was accompanied by massive cellular infiltration and extensive tissue damage whereas the skin grafts from control Ig treated Rag<sup>−/−</sup> mice were normal (Fig. 5). Staining for the NK1.1 marker by immunofluorescence method revealed abundant NK cells in the cellular infiltrates in rejecting skin allografts (Fig. 5c). Furthermore, transcription profile analysis of skin allografts harvested from IL-15-treated Rag<sup>−/−</sup> mice showed that the NK-related effector molecules were highly expressed as compared with the controls (Fig. 6). Taken together, these data suggest that IL-15 stimulated NK cells are the primary effector cells in mediating the rejection response in this model.

NK-mediated graft rejection lacks typical memory responses

Clearly, NK cells are capable of mediating skin allograft rejection, but this effect is contingent on their activation and further effector differentiation, a feature that is similar to that of T cells. There is evidence that NK cells also express features of memory cells, as NK cells can mount an Ag-specific recall response in the complete absence of T and B cells (30). This prompted us to examine whether IL-15-activated NK cells could mediate memory-like rejection in transplant models. For this purpose, we grafted a second DBA/2 skin allograft onto IL-15-treated Rag<sup>−/−</sup> mice that had rejected the primary DBA/2 graft 30 to 40 days earlier, and the skin allograft survival was determined. As shown in Fig. 7, all the animals that rejected the first skin allografts accepted the second DBA/2 skin allografts without any gross or histological signs of rejection. This finding suggests that NK cells do not acquire features of memory cells in this model and that NK-mediated rejection only occurs within a defined timeframe after activation. These
features are in striking contrast to that of adaptive T cells in transplant models (31). Phenotypic analysis of NK cells from recipient mice that accepted the second DBA/2 skin allografts showed a phenotype that is identical to resting NK cells in untreated control Rag−/− mice (Fig. 8a). We further analyzed the effector molecule granzyme B by intracellular staining at various times after IL-15 treatment. As shown in Fig. 8b, granzyme B expression was markedly up-regulated in NK cells 4 days after IL-15 treatment, and by day 80, however, its expression returned to the basal levels, suggesting that activated NK cells may have reverted to a resting state.

**Discussion**

The precise role of NK cells in the allograft response (rejection vs tolerance) remains enigmatic as NK cells have been implicated in both graft rejection and tolerance induction (3, 13, 32, 33). In the present study, we used the Rag−/− mice as a model system to critically examine the alloreactivity of NK cells in the absence of any adaptive immune cells and demonstrated several previously unknown features concerning NK cells in solid organ rejection. Apparently, MHC mismatch between donors and recipients is necessary but not sufficient for NK-mediated rejection of solid organ.

---

**FIGURE 5.** Histology and immunohistochemistry of rejection of skin allografts. *a,* Pictures shown are representative changes in skin allograft rejection in Rag−/− mice treated with or without IL-15/IL-15Ra complex. *b,* IL-15-treated Rag−/− and Rag−/−yc−/− mice were transplanted with DBA/2 skin allografts. Ten days later, the skin allografts were harvested and examined for tissue damage by H&E staining. Pictures shown are representative sections of three to five animals in each group. *c,* IL-15-treated Rag−/− mice were grafted with DBA/2 skin allografts and examined for NK infiltration in the skin allografts 5 days later by immunohistochemistry. Pictures shown are a representative section of three animals in each group.
transplants. MHC incompatibility produces alloreactive NK cells, which are cytolytic to allogeneic cells as shown by the rapid destruction of allogeneic but not syngeneic cells in vivo, but rejection of solid allografts demands further functional maturation of NK cells. In this regard, IL-15 plays a critical role in supporting NK activation and NK-mediated graft rejection. Clearly, neither Rag$^{-/-}$ nor Rag$^{-/-}\gamma c^{-/-}$ mice reject the DBA/2 skin allografts. Similarly, treatment of Rag$^{-/-}$ and Rag$^{-/-}\gamma c^{-/-}$ mice with IL-15/IL-15Rα complex also fails to elicit rejection of syngeneic B6 skin grafts. However, IL-15-treated Rag$^{-/-}$ mice (with functional NK cells), but not the Rag$^{-/-}\gamma c^{-/-}$ mice (NK deficient), promptly reject the DBA/2 skin allografts. It is noteworthy that NK cells in Rag$^{-/-}$ mice do not cause any damage to self skin after IL-15 treatment. These data suggest that both activation and IL-15-driven maturation of alloreactive NK cells are required for the rejection of the skin allografts in our model.

The requirement of NK cells in skin allograft rejection is somewhat reminiscent of that of T cells in other models. However, graft rejection by NK cells is strikingly different from that mediated by T cells. It seems that a complete rejection of skin allografts by activated NK cells is confined to a particular timeframe after NK activation. When NK cells fail to completely reject the graft within such a timeframe, the remaining skin allografts usually survive long-term. This is never the case in T cell-mediated rejection as destruction of skin allografts by activated T cells is always complete in the absence of anti-rejection therapies. The precise mechanisms by which the activated NK cells induce prompt skin allograft rejection remain to be defined. It is unclear whether NK-mediated skin allograft rejection is due to classical destruction of parenchymal skin cells or to inhibition of initial engraftment and revascularization of the transplanted skin grafts. Both scenarios may inhibit graft survival through continued activation of NK cells by missing self MHC I, but the mechanism and timing of graft rejection may be different. Nonetheless, both NK mediated cytolytic effect and NK-mediated tissue inflammation are likely involved in the rejection response. It is also unclear whether the activated NK cells undergo apoptotic cell death or they rapidly revert to a resting state following activation. More studies might be warranted to further unravel this issue. Another key feature of NK-mediated rejection is that NK cells fail to acquire properties of memory cells, even when they are optimally stimulated by IL-15. This is particularly intriguing because IL-15 is a key γc cytokine involved in the development and survival of memory T cells (34). This dichotomy further suggests a fundamental difference between NK cells and T cells in the rejection response. Interestingly, in the Rag$^{-/-}$ mice that rejected the first DBA/2 skin allograft but accepted the second one, NK cells can still reject allogeneic DBA/2 cells (our unpublished observation), suggesting that the alloreactive NK cells are not completely depleted in the recipient mice. This finding also suggests that the requirement of NK cells for rejection of allogeneic cells and allogeneic grafts may be completely different in transplant models.

Our data are the first to demonstrate that graft rejection is not exclusively confined to the adaptive immune cells, and NK cells by themselves can also trigger graft rejection when fully activated and differentiated under certain conditions. Our data expand the traditional concept of graft rejection and provides mechanistic insights on NK cells as effector cells in transplant models. The contribution of NK cells to graft rejection is likely dependent on the activation status of NK cells and the availability of NK-activating cytokines.
such as IL-15. This finding may have important clinical implications concerning the role of NK cells in autoimmunity and transplant tolerance. In a resting state, NK cells may actually promote transplant tolerance by killing graft-derived donor APCs (13). Under certain inflammatory conditions where IL-15 (possibly other NK stimulating factors) is produced at high levels, NK cells may undergo further activation and effector maturation, and then contribute significantly to graft rejection, and possibly also to the resistance to tolerance induction. As the role of NK cells as potent effector cells in transplant models is poorly studied and therapeutic strategies to modulate NK cells are also lacking, the models we have developed in this study may provide a valuable tool to further address these issues. Hopefully, studies in this area may lead to the design of greatly improved therapeutic strategies in tolerance induction in the clinic.

Acknowledgments
We thank Eva Csizmadia for kind help with tissue histology.

Disclosures
The authors have no financial conflict of interest.

References
NK CELLS IN TRANSPLANT REJECTION


