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Role of Natural Killer Cell Subsets in Cardiac Allograft Rejection

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To achieve donor-specific immune tolerance to allogeneic organ transplants, it is imperative to understand the cell types involved in acute allograft rejection. In wild-type mice, CD4$^+$ T cells are necessary and sufficient for acute rejection of cardiac allografts. However, when T-cell responses are suboptimal, such as in mice treated with costimulation-targeting agents or in CD28-deficient mice, and perhaps in transplanted patients taking immunosuppressive drugs, the participation of other lymphocytes such as CD8$^+$ T cells and NK1.1$^+$ cells becomes apparent. We found that host NK but not NKT cells were required for cardiac rejection. Ly49G2$^+$ NK cells suppressed rejection, whereas a subset of NK cells lacking inhibitory Ly49 receptors for donor MHC class I molecules was sufficient to promote rejection. Notably, rejection was independent of the activating receptors Ly49D and NKG2D. Finally, our experiments supported a mechanism by which NK cells promote expansion and effector function of alloreactive T cells. Thus, therapies aimed at specific subsets of NK cells may facilitate transplantation tolerance in settings of impaired T-cell function.

Key words: Costimulation, mouse, NK cells, tolerance, transplantation

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Introduction

Understanding acute allograft rejection is of critical importance for achieving successful organ transplant outcomes. In mice, CD4$^+$ T cells are necessary and sufficient for acute rejection of cardiac allografts (1–5). Although the CD4$^+$ response is attenuated by disruption of CD28-B7 interaction, CD28-deficient mice retain the ability to acutely reject cardiac allografts (6–8). This is due to a rejection pathway that, unlike in wild-type recipients, depends on CD4$^+$, CD8$^+$ T cells (8) and NK1.1$^+$ cells (9). However, the subset and mechanism by which NK1.1$^+$ cells promote rejection remains unknown. CD28$^{-/-}$ mice have normal numbers of NK and NKT cells. Whereas function of NK cells is normal (10), NKT cells may have impaired cytokine response to alpha-GalCer (11,12). Thus, either of these NK1.1$^+$ populations may contribute to rejection.

In this study, we found that NK cells and not NKT cells promote acute rejection of H-2^d cardiac grafts in H-2^b CD28$^{-/-}$ mice. A small subset of NK cells was sufficient to induce rejection and was regulated by the classical 'missing-self' response characteristic of NK cells. We have previously reported that H-2^b NK cell rejection of H-2^d bone marrow is dependent on the H-2D^d-binding activating receptor Ly49D (13). Herein, we determined that Ly49D was not required for the NK cell response to H-2^d cardiac transplants, suggesting that NK cell responses to allogeneic solid organs and bone marrow are markedly different. In addition, we demonstrate that NK cells promote proliferation and effector function of alloreactive T cells.

Materials and Methods

Mice

C57BL/6 (H-2^b), BALB/c (H-2^d), C57BL/6 X BALB/c (H-2^{dxb}), RAG-1$^{-/-}$ and CD28$^{-/-}$ (C57BL/6 background) mice were purchased from Jackson Laboratories. CD1$^{-/-}$/CD28$^{-/-}$ mice (C57BL/6 background) (14) and 2C TCR Tg RAG-1$^{-/-}$ were maintained at the University of Chicago. All experiments performed were approved by the University of Chicago Animal Care and Use Committee and according to NIH guidelines.

Antibodies

Antibodies were injected i.p. Anti-2B4 (20 μL ascitis, days −2 and +3); anti-asialo-GM1 (10 μL ascitis, Wako days −2, +3); anti-Ly49G2 (4D11, 150 μL ascitis, days −2, +1); anti-Ly49C/I (5E6, 100 μL ascitis, days −2, +1); anti-Ly49D (12A8, 200 μg, days −2, +1; or 4E5, 150 μg, days −2, +1); anti-NKG2D (CX5, eBioscience, 133 or 200 μg, days −1, 0, +4) (15). Subset

depletion and NKG2D blockade were always confirmed by PBL FACS analysis.

Ly49G2 (4D11) and Ly49A (YE1/32) mAbs were purified from hybridomas and fluorescently labeled (University of Chicago). Fluorescent-labeled mAbs purchased from BD Biosciences were anti-Ly49A (A1), anti-Ly49A/D (12A8), anti-Ly49C/I (5E6), anti-Ly49D (4E5), anti-Ly49I (YLI90), SA-PE, SA-APC and isotype controls. Purified and fluorescently labeled mAbs purchased from eBioscience were anti-NK1.1 (PK136), anti-NKG2D BIO (CX5) and anti-CD3 (145-2C11).

Cardiac transplantation

The abdominal heterotopic cardiac transplantation protocol was performed as previously described (16).

Flow cytometry

Flow cytometry was performed as previously described (17). For analysis of heart-infiltrating lymphocytes, cardiac transplants were rinsed *in situ* with HBSS/heparin, explanted, cut and digested with collagenase IV (Sigma-Aldrich, 400U/mL) and DNase I (MP Biomedicals, 0.01%) in HBSS for 40 min at 37°C. Mononuclear cells were isolated following density gradient centrifugation over a 44.5% Nycodenz solution, washed and analyzed by FACS.

Mixed lymphocyte reaction

C57BL/6 NK cells were derived by culture of RAG-$1^{-/-}$ splenocytes as described previously (17). On day 4, NK cells were plated with IL-2 in wells coated with anti-NK1.1 (1 μg/mL) for 2 days, washed extensively and added to MLR cultures.

T cells were purified from C57BL/6 lymph nodes by negative selection using MACS columns (Miltenyi Biotec). T cells were labeled with CFSE (Invitrogen, 1 μM, 10 min at 37°C). γ-irradiated (2500 rad) splenocytes (8×10^5/well) were used to stimulate responder T cells (2×10^5/well). NK cells were added at the indicated NK cell:T-cell ratios.

IFNγ ELISPOTs

ELISPOTs were performed as previously described (16).

Statistical analysis

Differences between mean survival times (MST) were compared using Kaplan-Meier/log rank test methods. Student's *t*-test compared T-cell functional readouts.

Results

Recipient classical NKT cells are not required for acute cardiac allograft rejection

It has been reported that $NK1.1^+$ cells are required for acute cardiac allograft rejection by CD28-deficient mice (9). NK1.1 is expressed on both NK and NKT cells (18–22). To test the role for host NKT cells in acute cardiac allograft rejection we used CD1-deficient mice, which lack classical NKT cells (14). As reported previously using anti-NK1.1 (9), depletion of NK and NKT cells using anti-2B4 (23) or anti-asialo-GM1 resulted in acceptance of most BALB/c cardiac grafts by $CD28^{-/-}$ recipients (Figure 1). In contrast, $CD1^{-/-}CD28^{-/-}$ mice acutely rejected heart transplants similar to $CD28^{-/-}$ recipients. Therefore, recipient NKT cells are not required for acute cardiac allograft rejection in $CD28^{-/-}$ mice. Anti-2B4 treatment did not deplete T cells, as T cells constituted 41 ± 3% of PBLs in control mice and 43 ± 9% in anti-2B4-treated mice.

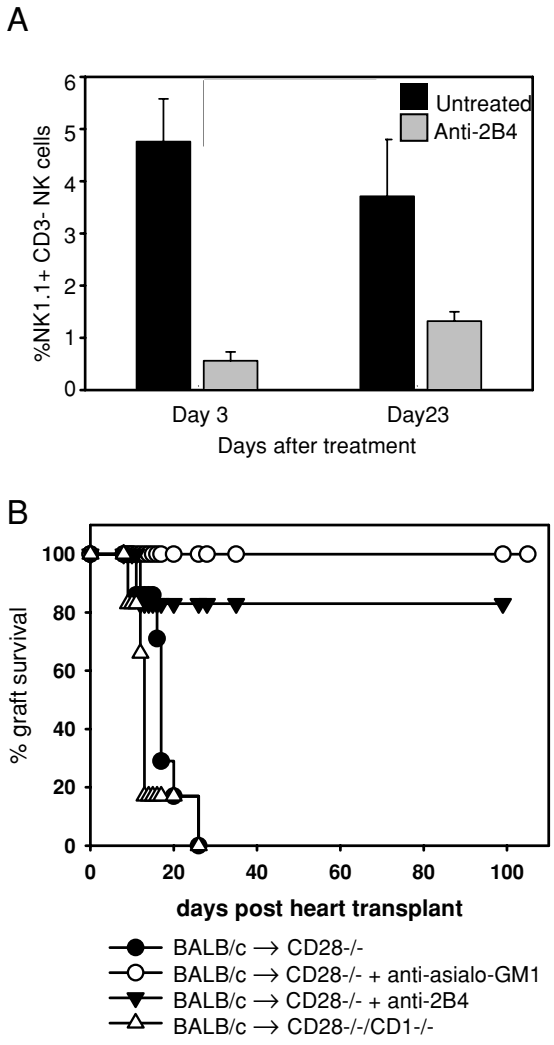

Figure 1: Classical NKT cells are not necessary for cardiac allograft rejection in $CD28^{-/-}$ mice. $CD28^{-/-}$ C57BL/6 mice were untreated (n = 7), or treated with anti-asialo-GM1 (n = 4), or anti-2B4 (n = 6) and transplanted with allogeneic BALB/c cardiac grafts. $CD1^{-/-}/CD28^{-/-}$ recipients (n = 6) also received BALB/c hearts. (A) The percentage of $NK1.1^+$ $CD3^-$ NK cells in the peripheral blood of untreated and anti-2B4-treated $CD28^{-/-}$ mice was measured on days 3 and 24 after treatment. (B) Allogeneic graft survival was monitored over time.

Semi-allogeneic cardiac grafts are accepted in $CD28^{-/-}$ mice

NK cells are regulated by MHC class I expression on target cells as described in the missing-self hypothesis (24). As such, allogeneic bone marrow transplants are eliminated by NK cells, but semi-identical F1 bone marrow is not rejected because of self-MHC coexpression (22). To confirm a role for NK cells in cardiac allograft rejection, we transplanted C57BL/6 $CD28^{-/-}$ mice with F1 semi-identical hearts. In agreement with a previous report (9), F1 cardiac transplants were accepted by $CD28^{-/-}$ mice but

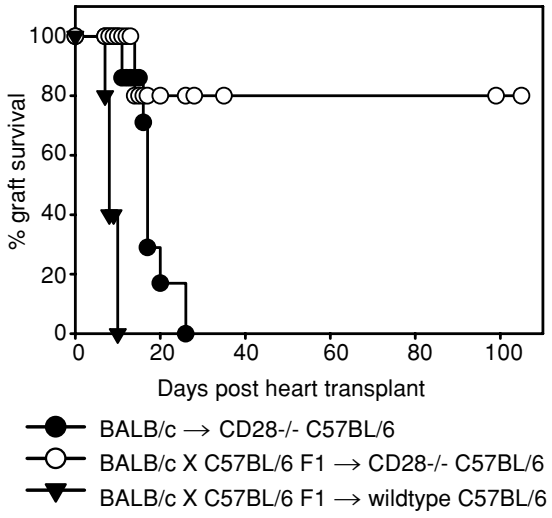

Figure 2: CD28$^{-/-}$ mice do not reject semi-identical H-2^{dxb} F1 cardiac grafts. C57BL/6 x BALB/c F1 hearts were transplanted into CD28$^{-/-}$ (n = 5) and wild-type (n = 5) C57BL/6 recipients. Control BALB/c donor hearts transplanted into CD28$^{-/-}$ C57BL/6 recipients (n = 7) are shown for comparison.

rejected by wild-type animals (Figure 2). Therefore, NK cells are required for acute cardiac allograft rejection in CD28-deficient mice.

Ly49D$^+$ Ly49G2$^-$ NK cells are recruited early to H-2^d allografts

To determine the subsets of NK cells that are recruited to cardiac grafts, allogeneic and syngeneic hearts were transplanted into CD28$^{-/-}$ hosts. Flow cytometry of heart-infiltrating leukocytes revealed a rapid recruitment of NK1.1$^+$/CD3$^-$ NK cells to allogeneic hearts, but not syngeneic controls (Figure 3A). NK cell infiltration peaked on day 6 post-transplant (Figure 3B), whereas CD3$^+$ T-cell infiltration did not peak until at least 8 days post-transplantation (Figure 3A,B). Similar results were found in allografts in wild-type hosts (data not shown). Consistent with our finding that host NKT cells are not required for graft rejection, we did not observe a significant population of NK1.1$^+$/CD3$^+$ NKT cells recruited to allogeneic transplants (Figure 3A).

Murine NK cells express inhibitory and activating Ly49 receptors that recognize polymorphic epitopes of MHC class I molecules (25). Ly49s are expressed on partially overlapping subsets of NK cells. We hypothesized that rejection of H-2^d heart transplants is mediated by a subset of NK cells expressing activating receptors for H-2^d (Ly49D) and lacking inhibitory receptors for H-2^d (Ly49A, Ly49C and Ly49G2) (26), as we have reported in H-2^d bone marrow transplant rejection (13). To characterize the subset of NK cells involved in cardiac allograft rejection we examined the Ly49 repertoire on cardiac-infiltrating NK cells. The low frequency of NK cells within syngeneic transplants hindered conclusive assessment of Ly49 receptor expression (Figure 3C). In allogeneic hearts, a higher proportion of NK cells were Ly49D$^+$ on day 6 post-allogeneic transplantation when compared with splenic NK cells (Figure 3C). In contrast, NK cells expressing inhibitory receptors for H-2D^d were underrepresented in cardiac allografts with fewer than 6% of NK cells expressing Ly49G2, compared to 30% in the spleen. Ly49C/I/A were also expressed on a lower fraction of allograft infiltrating NK cells than in the spleen, although differences were less dramatic.

We have previously shown that 57% of Ly49D$^+$ NK cells normally coexpress Ly49G2 (13). Within allograft-infiltrating NK cells, 35% expressed Ly49D, while only 5.8% expressed Ly49G2 (Figure 3C). Thus at most, 16% of Ly49D$^+$ allograft-infiltrating NK cells coexpressed Ly49G2. This indicates that of the Ly49D$^+$ NK cells, those that lack Ly49G2 expression are preferentially recruited to the allografts, or that Ly49G2 expression is down-regulated in the transplants. This profile was consistent with the hypothesis that the NK cell population involved in H-2^d organ rejection expresses activating receptors for H-2^d and low levels of inhibitory receptors for H-2^d.

Depletion of cells bearing H-2^d-binding inhibitory receptors accelerates cardiac graft rejection

To identify the subset of NK cells required for cardiac rejection, we first tested the hypothesis that NK cells bearing H-2^d-binding inhibitory receptors do not have a role in rejection. CD28$^{-/-}$ mice were treated with anti-Ly49G2 and anti-Ly49C/I to deplete cells expressing this receptor (Figure 4A). The majority of Ly49A$^+$ cells also expresses Ly49G2 or Ly49C/I (27), therefore Ly49A$^+$ cells were also depleted by these antibodies. Although 80% of Ly49D$^+$ cells also express Ly49G2 or Ly49C/I and are thus depleted by this treatment, the remaining Ly49D$^+$ cells are sufficient for rejection of BALB/c bone marrow transplants (28,29), and may likewise be sufficient for cardiac rejection.

Mice depleted of Ly49G2/C/I$^+$ cells acutely rejected cardiac allografts (Figure 4B), confirming the hypothesis that NK cells bearing H-2^d-binding inhibitory receptors are not required for rejection. Interestingly, mice depleted of Ly49G2/C/I$^+$ cells not only rejected cardiac allografts acutely but did so with significantly faster kinetics than untreated recipients (Figure 4B) (MST 13 $\pm$ 05 vs. 19 $\pm$ 1.5 days, $p = 0.013$). This suggests that the population of cells expressing this H-2^d-binding inhibitory receptor suppresses H-2^d graft rejection in CD28-deficient mice, and depletion of these cells leads to accelerated rejection. In fact, depletion of Ly49G2$^+$ cells alone resulted in even faster acceleration of rejection (MST 9.5 $\pm$ 1, $p = 0.008$ when compared to untreated CD28$^{-/-}$ mice and when compared to Ly49G2/C/I-depleted mice, $p = 0.037$) (Figure 4B). This result strongly suggests that the Ly49G2$^+$ NK cell subset contains cells capable of suppressing immune responses.

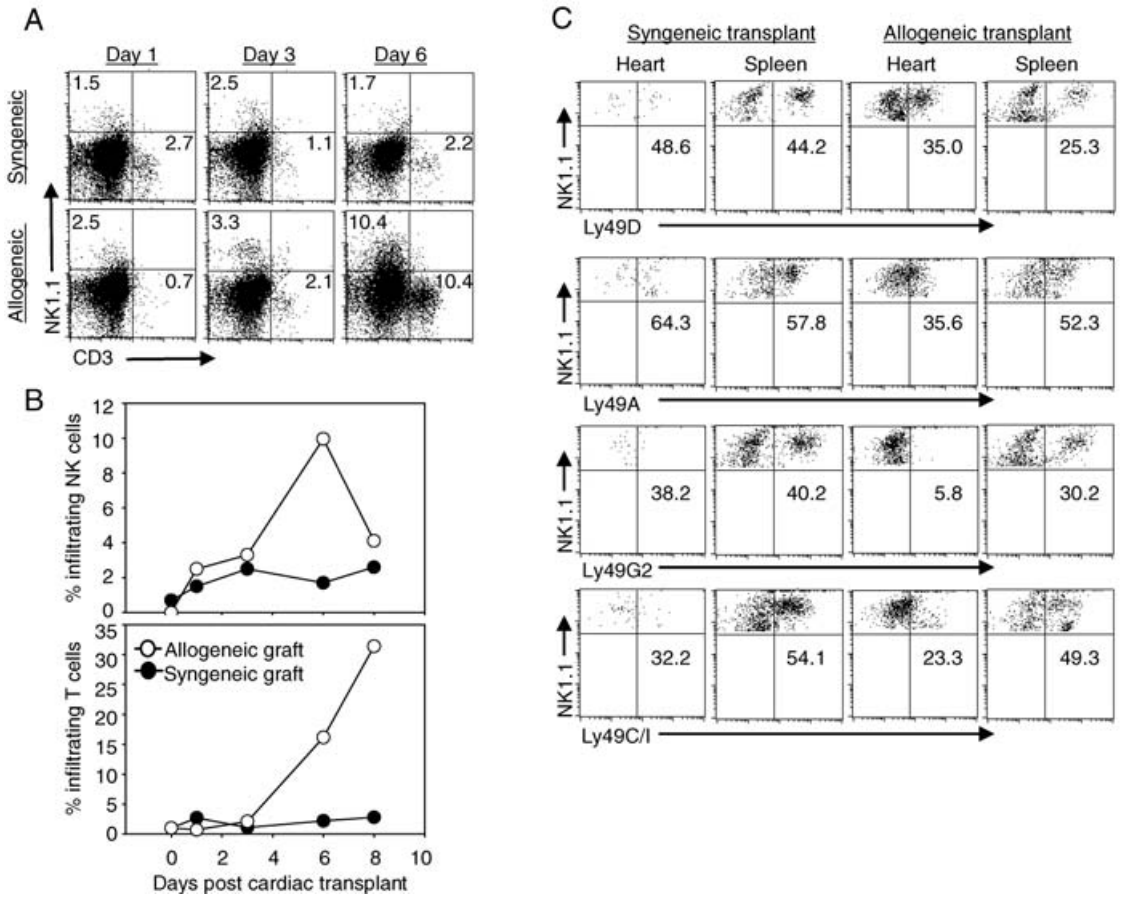

Figure 3: Ly49D$^+$ Ly49G2$^-$ NK cells infiltrate allogeneic cardiac grafts early post-transplantation. CD28$^{-/-}$ C57BL/6 recipients were transplanted with syngeneic or allogeneic BALB/c hearts. (A) Grafts from days 1, 3 and 6 post-transplant were collected and infiltrating leukocytes were analyzed for NK1.1 and CD3 expression by flow cytometry. Numbers indicate the percentage of cells within each quadrant. (B) The number of infiltrating NK cells (NK1.1$^+$ CD3$^-$ cells, top panel) and T cells (CD3$^+$ cells, lower panel) within syngeneic and allogeneic cardiac grafts is depicted over time. (C) On day 6 post-transplantation, graft-infiltrating leukocytes and host splenocytes were analyzed by flow cytometry. Cells were gated on NK1.1$^+$/CD3$^-$ NK cells and Ly49 receptor expression was determined. Numbers indicate the percentage of Ly49$^+$ NK cells for each Ly49 receptor. Leukocytes from 2 grafts per experimental group were pooled for analysis, and data are representative of 2 independent experiments.

In contrast, depletion of Ly49C/I alone did not have a significant effect on survival (data not shown). We cannot exclude a role for NKT cells in this negative regulation as Ly49G2 is also found on 10% of NKT cells (30); however, this is unlikely given the normal rejection kinetics in CD1$^{-/-}$ mice.

The NK cell activating receptors Ly49D and NKG2D are not essential for allograft rejection

To identify the NK cell activating receptor required for cardiac allograft rejection, we tested the role for Ly49D-bearing cells. In addition to Ly49D$^+$ cells, anti-Ly49A/D (12A8) depletes an additional 13% of NK cells that express Ly49A (31) and NK cells that coexpress Ly49A/D with other Ly49 receptors [72% of Ly49G2$^+$ NK cells and 60% of Ly49C/I$^+$ NK cells (32)]. Regardless of the partial depletion of other NK cell subsets, removal of the activating Ly49D$^+$ subset leads to acceptance of BALB/c bone marrow transplant (13,31). As shown in Figure 5A,B, depletion of Ly49D$^+$ cells did not delay allograft rejection in CD28$^{-/-}$ mice. Thus, our finding is in contrast to the requirement for Ly49D$^+$ NK cells in H-2^d bone marrow graft rejection (13) and suggests an alternative mechanism for NK cell activation by solid organ allografts.

NKG2D is an activating receptor expressed by all NK cells, including Ly49D$^+$ NK cells (33). NKG2D ligands are stress-induced, and are up-regulated on transplanted renal and pancreatic allografts (33,34). Accordingly, we tested the role for NKG2D on NK cells by treating CD28$^{-/-}$ mice with anti-NKG2D blocking antibody at doses sufficient for *in vivo* blockade of NKG2D function (35). Although NKG2D receptors were completely blocked on peripheral blood NK

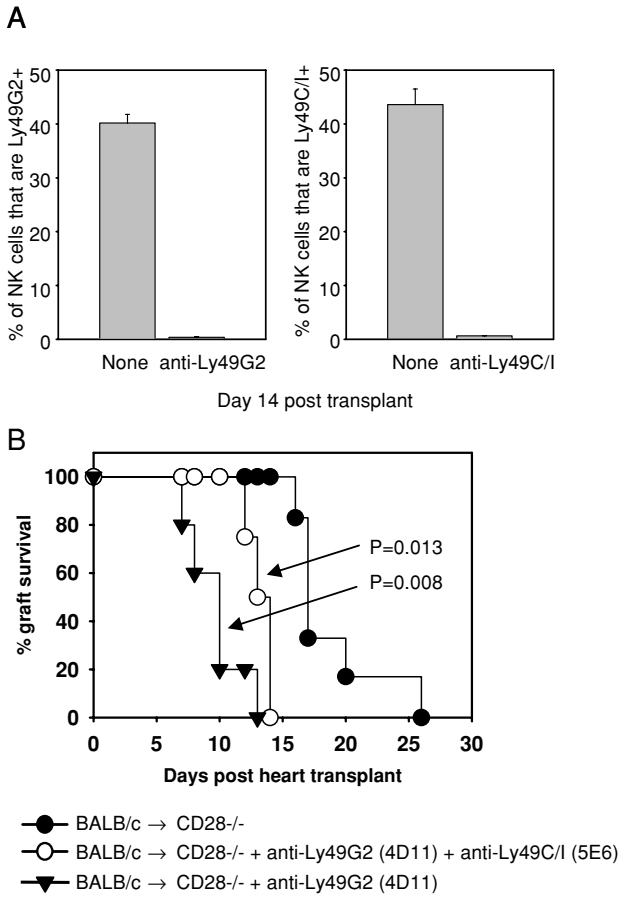

Figure 4: Depletion of Ly49G2$^+$ cells accelerates cardiac allograft rejection. CD28$^{-/-}$ mice were untreated or treated with anti-Ly49G2$^+$ and anti-Ly49C/I. (A) The percentage of NK1.1$^+$ CD3$^-$ NK cells that are Ly49G2$^+$ and Ly49C/I$^+$ in the peripheral blood was measured by flow cytometry on day 14 post-transplant. (B) Untreated (n = 6), anti-Ly49G2 (n = 5) and anti-Ly49G2$^+$ plus anti-Ly49C/I treated (n = 4) CD28$^{-/-}$ hosts were transplanted with allogeneic BALB/c cardiac grafts and graft survival was monitored.

cells (Figure 5E), mice treated with anti-NKG2D rejected allografts at a similar rate as untreated mice (Figure 5C), demonstrating that NKG2D ligation is not required for NK cell-mediated graft rejection.

It was plausible that NKG2D and Ly49D acted redundantly in NK cell allograft responses and that abrogation of both was required to block NK cell activation. CD28-deficient mice were treated with anti-Ly49D (4E5) to deplete Ly49D$^+$ cells and anti-NKG2D blocking antibody. Even in the absence of both Ly49D and NKG2D receptor function, there was no delay in the rejection of cardiac allografts by CD28$^{-/-}$ mice (Figure 5D). These data indicate that NK cell-mediated rejection of allogeneic solid organ transplants involves a novel mechanism of NK cell activation that occurs independently of Ly49D and NKG2D.

NK cells augment T-cell proliferation and effector function

We have previously determined that T cells are required for acute cardiac rejection in CD28$^{-/-}$ hosts (8), implying that NK cells alone are not sufficient for rejection. The finding that NK cells are necessary, but not sufficient, for cardiac allograft rejection is consistent with a role for NK cells in providing help to T cells, which may be important in the absence of CD28-dependent T-cell costimulation. To test this hypothesis, we designed an MLR that reflects the *in vivo* transplant setting. CFSE-dyed C57BL/6 responder T cells were cultured with irradiated BALB/c stimulator cells. The ability of added activated NK cells to enhance T-cell activation was measured by T-cell CFSE dilution.

Addition of NK cells augmented the percentage of T cells that divided at least once in response to allogeneic stimulator cells (Figure 6A). Similar results were seen using 2C TCR Tg T cells that recognize L^d, and thus have an increased frequency of responding T cells (Figure 6B). On average, the addition of NK cells to the MLR caused a 2-fold increase in the number of T cells that proliferated (Figure 6B).

To determine if NK cells promote T-cell responses *in vivo*, CD28$^{-/-}$ mice were depleted of NK cells and transplanted with cardiac allografts. Animals were sacrificed 3 weeks later and splenocytes were restimulated *in vitro* with syngeneic or donor irradiated splenocytes. NK depletion *in vivo* resulted in reduced frequency of IFNγ-producing cells upon restimulation *in vitro* (Figure 6C), suggesting that NK cells help the priming and/or differentiation of alloreactive T cells during a transplant response.

Discussion

We have found that host NK and not NKT cells contribute to cardiac allograft rejection in CD28$^{-/-}$ mice. Allograft-infiltrating NK cells are enriched in cells expressing activating receptors for H-2^d and low levels of inhibitory receptors for H-2^d. Ly49G2$^+$ NK cells suppress alloresponses, whereas a subset of NK cells lacking expression of H-2^d-binding inhibitory Ly49 receptors is sufficient to promote cardiac rejection. Surprisingly, rejection was independent of the activating receptors NKG2D and Ly49D. Finally, unseparated NK cells may be promoting alloreactive T-cell function.

NK cell responses to allogeneic bone marrow and cardiac transplants have important commonalities. In both settings, NK cells are regulated by the missing-self response (Figures 1 and 2) and the subsets of NK cells inhibited by donor MHC do not mediate rejection (Figure 4) (22). However, NK cell activation in response to cardiac allografts was unexpectedly different in that it is not dependent on Ly49D (Figure 5). This suggests that distinct mechanisms trigger NK cell rejection of bone marrow and solid organ

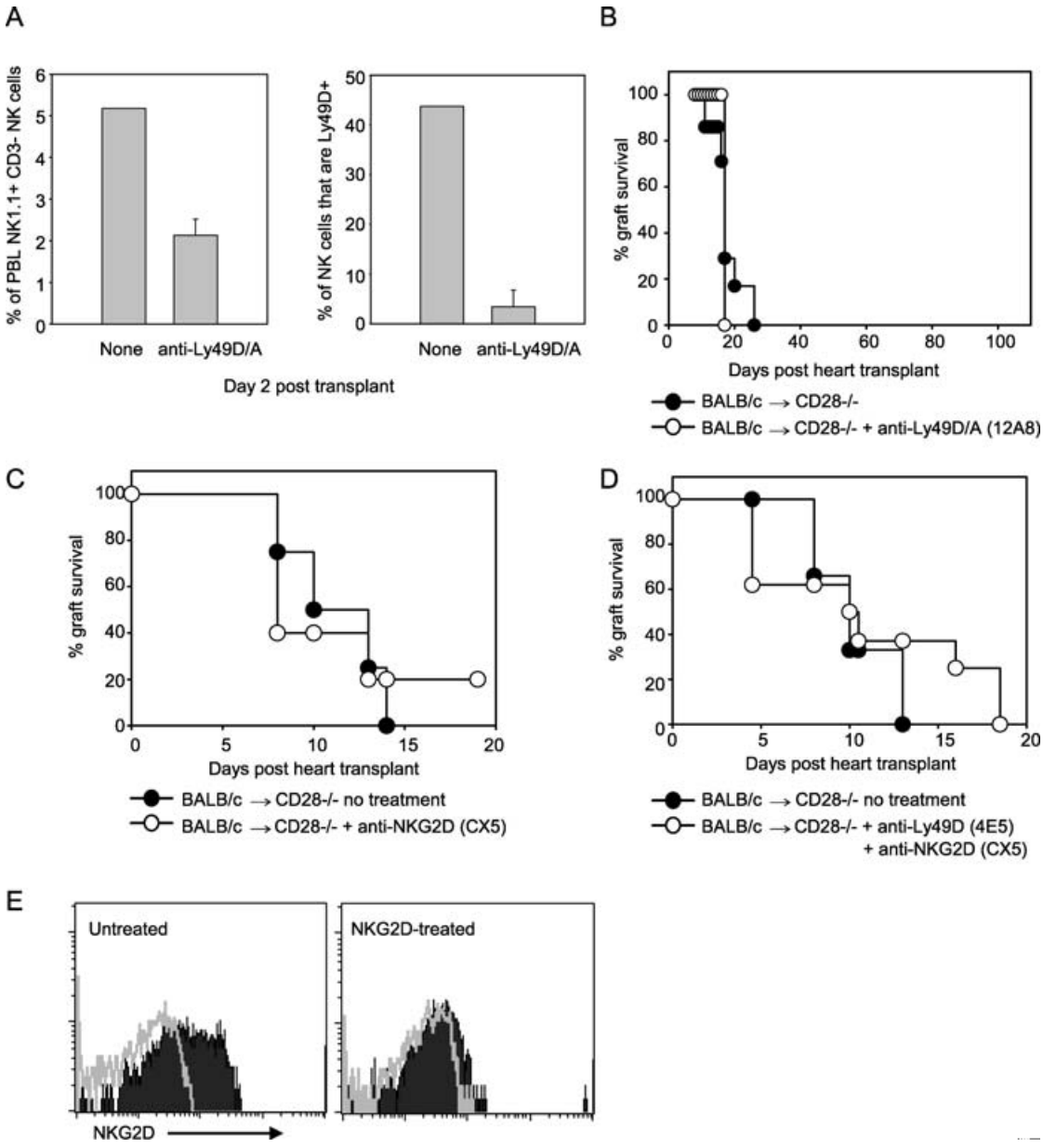

Figure 5: The NK cell activating receptors Ly49D and NKG2D are not essential for NK cell-dependent cardiac allograft rejection. (A) $CD28^{-/-}$ mice were untreated or treated with anti-Ly49D/A. The percentage of $NK1.1^{+}/CD3^{-}$ NK cells in the peripheral blood was measured on day 2 post-transplant. The percentage of $Ly49D^{+}$ NK cells is also indicated. (B) Untreated (n = 7) and anti-Ly49A/D treated (n = 6) $CD28^{-/-}$ hosts were transplanted with allogeneic BALB/c cardiac grafts and graft survival was monitored. (C) $CD28^{-/-}$ mice were untreated or treated with non-depleting blocking anti-NKG2D (133 μg days –1, 0 and 4; n = 5). Mice were transplanted with BALB/c allogeneic cardiac grafts and graft survival was monitored. (D) $CD28^{-/-}$ mice were untreated or treated with both anti-Ly49D (4E5, 150 μg days –2 and +1) and anti-NKG2D (200 μg days –1, 0 and +4) (n = 8). Mice transplanted with BALB/c allogeneic cardiac allografts were monitored for graft survival. (E) NKG2D expression on peripheral blood $NK1.1^{+}/CD3^{-}$ cells in an untreated (left panel) and anti-NKG2D-treated mouse (right panel) on day 11 post-transplant. Open histogram indicates isotype control and closed histogram indicates NKG2D staining.

transplants. Although $Ly49D^{+}$ NK cells are not essential, we have not excluded the possibility that they participate in rejection. Another subset of NK cells may redundantly mediate rejection in the absence of $Ly49D^{+}$ cells, masking a role for $Ly49D^{+}$ NK cells recruited to cardiac allografts (Figure 3). Similarly, we have previously found $CD8^{+}$ T cells to be recruited to cardiac allografts despite not being required for rejection in wild-type hosts (8). $CD8^{+}$ T cells may still

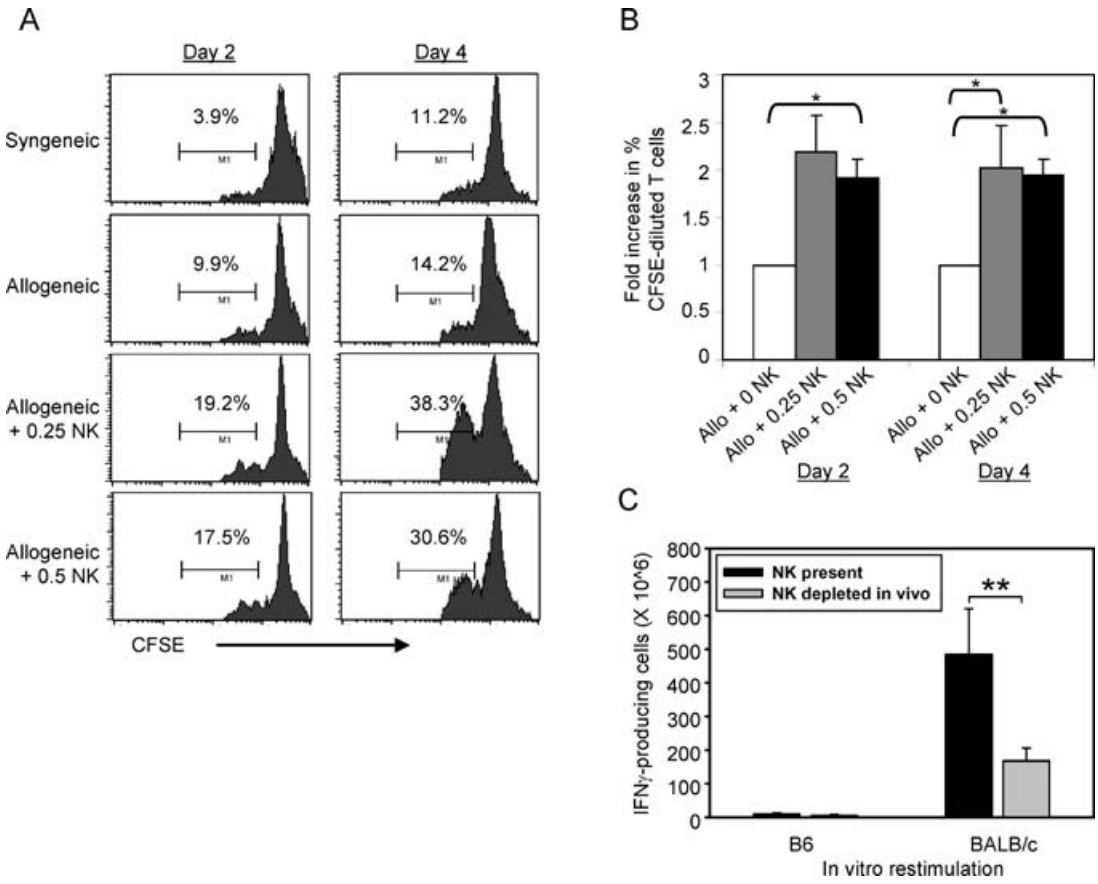

Figure 6: A small number of NK cells augment T-cell effector functions in an MLR. Activated NK cells from C57BL/6 mice were added to MLRs consisting of either wild type or 2C TCR Tg C57BL/6 T cells dyed with CFSE, and allogeneic BALB/c stimulator splenocytes at the indicated NK:T-cell ratios. (A) Cultures were analyzed on days 2 and 4 for $CD3^+/CFSE^+$ T cells. The percentage of wild-type $CFSE^+$ T cells that underwent at least one division is shown. Data represent one of three independent experiments. (B) Mean + SD from 3 independent experiments, as depicted in panel A, normalized by dividing by the percentage of CFSE-diluted T cells in the absence of NK cells in each experiment (2 experiments with wild type and 1 with 2C TCR Tg T cells; $*p < 0.05$). C. $CD28^{-/-}$ mice were treated with anti-2B4 to deplete NK cells ($n = 4$) or left untreated ($n = 3$) and transplanted with BALB/c cardiac allografts. Animals were sacrificed 3 weeks later and splenocytes were restimulated *in vitro* with syngeneic or donor irradiated splenocytes. The percentage of IFNγ-producing cells upon restimulation was measured by ELISPOT ($**p < 0.01$).

participate in rejection—in fact in $CD28^{-/-}$ hosts the role for $CD8^+$ T cells in rejection is apparent (8).

NKG2D regulates the NK cell response to tumors and infections (34). However, we ruled out the possibility that NKG2D, either acting alone or in concert with Ly49D, triggers cardiac rejection. Compared to inhibitory receptors, NK cell activating receptors on the whole are less well characterized, thus the receptor-ligand pair that triggers NK cell rejection of cardiac allografts may be difficult to identify. It has recently been reported that KLRE-1 (NKG2I) is required for NK cell rejection of $H\text{-}2^d$ bone marrow transplants (36). While the ligand for NKG2I remains unknown, it may play a role in cardiac rejection.

We predicted that depletion of host NK cells that are inhibited by donor MHC would not alter the course of rejection. Unexpectedly, this depletion led to accelerated allograft rejection. A similar finding has also been observed in bone marrow rejection (29). These results may reflect a tolerizing capacity for NK cells. Our results indicate that it is the $Ly49G2^+$ NK cell subset that contains the capacity to suppress alloresponses. Interestingly, although $Ly49G2^+$ cells promoted graft acceptance, they were underrepresented within the allograft (Figure 3). One explanation for this discrepancy is that $Ly49G2^+$ NK cells residing within the graft have internalized Ly49G2 receptors post-engagement with MHC-ligands causing their decreased detection. Alternatively, inhibitory NK cells may not be acting within the graft, but in secondary lymphoid organs. The mechanism for such inhibition is unknown, though a recent publication has found that NK cells promote islet allograft tolerance in anti-CD154-treated mice in a perforin-dependent mechanism (37). It was proposed that inhibitory NK cells may

promote tolerance by lysing donor DCs and/or alloreactive T cells.

To begin to elucidate the mechanism by which NK cells promote cardiac allograft rejection, we determined that NK cells could enhance antigen-specific T-cell proliferation and IFNγ production. This finding points to a role for NK cells in providing help to $CD28^{-/-}$ T cells. NK cell support may take several, non-exclusive routes. A number of studies have reported NK cell activation of APCs (38). Thus in transplant rejection, NK cells may likewise promote DC maturation and subsequent T-cell activation, overcoming the CD28-deficiency. Alternatively, NK cells may act directly on T cells through soluble factors or contact-dependent mechanisms (39,40). A third possibility is that NK cells may be causing tissue destruction within the cardiac grafts by producing chemokines that further recruit immune cells (41) and/or by directly damaging allogeneic endothelial cells (42,43).

In conclusion, our findings indicate that cardiac allograft transplant outcomes are regulated by NK cells. In agreement with this finding, humans with killer cell immunoglobulin-like receptors that are inhibited by donor MHC have a decreased risk of liver transplant rejection (44). In the case of renal transplantation, NK cells are not suppressed by current immunosuppressive treatments (45). With the emergence of clinical trials testing costimulation-targeting regimens in transplant patients, the role of NK cells in solid organ transplant rejection deserves reevaluation.

Acknowledgments

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