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In the Absence of Natural Killer Cell Activation Donor-Specific Antibody Mediates Chronic, But Not Acute, Kidney Allograft Rejection

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Abstract

Antibody mediated rejection (ABMR) is a major barrier to long-term kidney graft survival. Dysregulated donor-specific antibody (DSA) responses are induced in CCR5-deficient mice transplanted with complete MHC-mismatched kidney allografts, and natural killer (NK) cells play a critical role in graft injury and rejection. We investigated the consequence of high DSA titers on kidney graft outcomes in the presence or absence of NK cell activation within the graft. Equivalent serum DSA titers were induced in CCR5-deficient B6 recipients of complete MHC mismatched A/J allografts and semi-allogeneic (A/J x B6) F1 kidney grafts, peaking by day 14 post-transplant. A/J allografts were rejected between days 16–28, whereas B6 isografts and semi-allogeneic grafts survived past day 65. On day 7 post-transplant, NK cell infiltration into A/J allografts was composed of distinct populations expressing high and low levels of the surface antigen NK1.1, with NK1.1_{low} cells reflecting the highest level of activation. These NK cell populations increased with time post-transplant. In contrast, NK cell infiltration into semi-allogeneic grafts on day 7 was composed entirely of NK1.1_{high} cells that decreased thereafter. On day 65 post-transplant the semi-allogeneic grafts had severe interstitial fibrosis, glomerulopathy, and arteriopathy, accompanied by expression of pro-fibrogenic genes. These results suggest that NK cells synergize with DSA to cause acute kidney allograft rejection, whereas high DSA titers in the absence of NK

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DISCLOSURE

All the authors declared no competing interests.

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cell activation cannot provoke acute ABMR but instead induce the indolent development of interstitial fibrosis and glomerular injury that leads to late graft failure.

Keywords

kidney allograft; antibody-mediated rejection; NK cells

INTRODUCTION

The incidence of antibody-mediated rejection (ABMR) of solid organ transplants to treat end-stage organ disease increasing and antibodies are an important cause of the acute and chronic injury that leads to late graft failure and undermines graft outcomes (1–5). ABMR is initiated by donor-specific antibody (DSA) binding to target alloantigens, such as donor class I or class II MHC molecules, on the graft vascular endothelium. Antibody binding to allogeneic MHC targets induces their association with integrins that transduce intracellular signals to stimulate endothelial cell activation, including increased expression of adhesion molecules and production of proinflammatory cytokines (6–9). A common diagnostic feature of antibody-mediated injury is the detection of complement split products, C3d and C4d, on the large vessels and capillaries of the transplant indicating antibody binding to the endothelium followed by complement activation (10–13). Collectively, these activation events promote trafficking of graft recipient leukocyte populations, including neutrophils, macrophages and Natural Killer (NK) cells, to the graft and interaction with the vasculature.

How these leukocytes function in mediating or exacerbating ABMR remains incompletely understood. Recent studies have indicated that early and late rejection of kidney transplants are distinguished by distinct biopsy gene expression profiles, with early rejection accompanied by expression of genes associated with T cell mediated rejection and later rejection expression of genes associated with antibody-mediated injury that includes NK cell-related transcripts (14). Whether NK cells are activated within allografts during acute and/or chronic antibody-mediated graft injury and the impact of DSA on graft injury in the absence of NK cell activation is not well defined.

We previously reported the dysregulated DSA response elicited in CCR5^{-/-} recipients of vascularized complete MHC mismatched heart and kidney allografts (15–18). DSA elicited in CCR5-deficient kidney allograft recipients is first detectable on day 7 post-transplant and by day 14 titers are 40–100-fold higher than those elicited in wild type recipients. Acute rejection of kidney allografts in CCR5^{-/-} recipients requires DSA production as B cell depletion beginning at the time of transplant prevents rejection. The expression of genes encoding NK cell related transcripts in the kidney allografts led us to test the role of NK cells during acute ABMR of kidney allografts in CCR5^{-/-} recipients, where NK cell depletion abrogated acute rejection, suggesting a direct role for NK cells in ABMR of kidney allografts (19). In the current study, we tested NK cell activation within kidney allografts in CCR5^{-/-} recipients and the consequence of high titers of DSA on kidney graft outcomes in the presence versus the absence of NK cell activation.

RESULTS

Gating strategy to identify graft infiltrating NK cells in kidney allograft

The impact of NK cell infiltration and DSA on kidney graft outcome was investigated in B6.CCR5^{-/-} recipients where the remaining native kidney is removed on day 4 post-transplant and recipient survival depends on the kidney transplant function. In B6.CCR5^{-/-} recipients of complete MHC mismatched A/J kidney allografts DSA is first detected on day 7 post-transplant and reaches peak titers on day 14 (16, 19). NK cell infiltration and activation in A/J kidney allografts in B6.CCR5^{-/-} recipients and into isografts in wild type C57BL/6 recipients was assessed on days 7 and 14 post-transplant. Grafts were harvested, digested to prepare single cell suspensions, and aliquots stained with anti-CD3e, anti-NK1.1 and anti-CD49b (DX5) mAb and analyzed by flow cytometry. The gating strategy for identifying graft infiltrating NK cells on day 14 post-transplant is shown in Figure 1A. After eliminating doublet cells, the lymphocyte population was gated by forward (FSC) and side (SSC) scatter and then into CD3e⁻ cells to exclude T cells. Within the CD3e⁻ population, NK cells were identified as NK1.1^{high}DX5⁺ and NK1.1^{low}DX5⁺ subpopulations. On day 7 post-transplant, NK1.1^{high}DX5⁺ cell numbers were approximately 2-fold greater than NK1.1^{low}DX5⁺ cells in allografts and few NK cells of either phenotype were observed in isografts (Figure 1B). Each NK cell subpopulation was also analyzed for expression of CD107a that indicates exocytosis of perforin/granzyme containing granules as a marker of NK cell functional activity and a greater percentage of NK1.1^{low}DX5⁺ cells expressed CD107a than NK1.1^{high}DX5⁺ cells on day 14 post-transplant (Figure 1A). Allograft infiltrating NK1.1^{high}DX5⁺ and NK1.1^{low}DX5⁺ cell populations were also distinguishable by increased side scatter of NK1.1^{low}DX5⁺ versus the NK1.1^{high}DX5⁺ cells (Figure 1C). In contrast to the allograft infiltrating NK cells, NK cells in the spleens of naïve B6 and B6.CCR5^{-/-} kidney allograft recipients on days 7 and 14 post-transplant were mostly NK1.1^{high}DX5⁺ cells with few NK1.1^{low}DX5⁺ cells (Figure 1D and data not shown).

Graft infiltrating NK cells and functional activity in complete MHC mismatched A/J allograft and (A/J x B6) F1 semi-allogeneic allograft

Allograft infiltrating NK cells were detectable as early as day 7 in A/J kidney allografts and increased with time post-transplant (Figure 2A). When assessed on day 7 post-transplant absolute numbers of NK1.1^{high} cells within kidney allografts were higher than NK1.1^{low} cells but they were equivalent by day 14 (Figure 2B and C). NK cell CD107a expression appeared as early as day 7 post-transplant and although a greater percentage of NK1.1^{low} cells expressed the activation marker, the total numbers of NK1.1^{high} cells and NK1.1^{low} cells expressing CD107a were similar in the allograft (Figure 2D-E). NK cell infiltration into isografts and CD107a expression was low/absent.

NK cells express surface activating and inhibitory receptors and engagement of self class I MHC transduces dominant inhibitory signals that attenuate NK cell activation (20, 21). Therefore, we postulated that expression of recipient class I MHC with allogeneic class I MHC on kidney grafts would prevent NK cell activation during ABMR. We crossed the allogeneic donor A/J and recipient background C57BL/6 mice to generate (A/J x B6)F1 semi-allogeneic graft donors for B6.CCR5^{-/-} recipients and NK cell infiltration and activity

in the F1 semi-allogeneic kidney grafts was examined. NK cell infiltration into F1 kidney grafts was observed on day 7 post-transplant at numbers equivalent to those observed infiltrating complete MHC-mismatched A/J kidney allografts. In contrast to the NK cells infiltrating the A/J allografts, all NK cells infiltrating the semi-allogeneic F1 grafts on day 7 post-transplant were entirely NK1.1^{high} cells with few NK1.1^{low} cells (Figure 2A-C) and very few expressed CD107a (Figure 2D-F). Moreover, NK cell numbers in F1 grafts fell to near baseline/isograft numbers by day 14 post-transplant. Overall these results indicated increasing infiltration, accumulation and activation of NK cells into complete MHC-mismatched kidney allografts and early post-transplant infiltration of NK cells into semi-allogeneic grafts that decreased after day 7 post-transplant.

NK cell proliferation in complete MHC mismatched A/J allografts

Since NK1.1^{high} and NK1.1^{low} cells increased over time post-transplant in A/J allografts, we tested their proliferation within the allografts using BrdU labeling (Figure 3A). The total number of BrdU⁺ NK cells increased from day 7 to 14 post-transplant (Figure 3B), with no apparent difference in the percentage of proliferating NK1.1^{high} and NK1.1^{low} cells (Figure 3C). While the number of BrdU⁺ NK1.1^{low} cells within allografts was significantly lower than that of BrdU⁺ NK1.1^{high} cells on day 7 post-transplant, these increased to equivalent numbers as BrdU⁺ NK1.1^{high} cells by day 14 (Figure 3D). These results suggested that the increased number of NK1.1^{low} cells from day 7 to 14 post-transplant was due, at least in part, to the proliferation of NK1.1^{low} cells within the allograft.

NK1.1 is a member of the NKPR1 family of receptors on NK and NKT cells and NK1.1 expression is associated with Fcγ (22, 23), raising the possibility that FcγRIII/CD16 expression might be down regulated on NK1.1^{low} cells in kidney allografts during ABMR in B6.CCR5^{-/-} recipient mice. However, NK1.1^{low} cells expressed markedly higher CD16 than NK1.1^{high} cells in allografts (Figure 4). Furthermore, the NK1.1^{high} cells in the spleens of naïve C57BL/6 and allograft B6.CCR5^{-/-} recipient mice on day 7 post-transplant expressed low levels of CD16, but this expression increased on the NK1.1^{high} cells in the spleen of kidney allograft recipients by day 14 post-transplant.

Survival of F1 semi-allogeneic kidney allografts and induction of DSA

We previously reported that NK cell depletion abrogated acute ABMR of kidney allografts in B6.CCR5^{-/-} recipients (19). The absent NK cell activation in semi-allogeneic F1 kidney grafts led us to assess their survival in B6.CCR5^{-/-} recipients (Figure 5A). Consistent with our previous reports (16, 18, 19), A/J kidney allografts were rejected by B6.CCR5^{-/-} recipients within 30 days and approximately 70% rejected by day 20. In contrast, all F1 grafts survived more than 65 days in B6.CCR5^{-/-} recipients.

Since A/J kidney allograft rejection in B6.CCR5^{-/-} recipients requires DSA production (16, 18), we compared serum DSA titers in B6.CCR5^{-/-} recipients of A/J and F1 grafts. Identical peak DSA titers were reached by day 14 post-transplant in response to both A/J and F1 grafts and these titers were maintained in recipients of the A/J allografts until rejection between days 16 and 28 and in recipients of F1 semi-allogeneic grafts to day 65 when the grafts were harvested for analyses (Figure 5B). Titers of DSA in wild type C57BL/6

recipients of A/J allografts were approximately 100-fold lower than those elicited in B6.CCR5^{-/-} recipients of A/J and F1 grafts.

Histological evaluation of functioning F1 semi-allogeneic kidney graft from B6.CCR5^{-/-} recipients

Despite the high DSA titers, all F1 kidney grafts maintained function beyond 60 days post-transplant in B6.CCR5^{-/-} recipients. However, on day 20 post-transplant, F1 grafts had mononuclear cell infiltration that was most intense around arteries and glomeruli with little evidence of collagen deposition (Figure 6A) and marked deposition of C4d, used as an indication of antibody-mediated complement activation, in the peritubular capillaries and glomeruli of the semi-allogeneic kidney grafts (Figure 6B). Cellular infiltrates contained CD3⁺ T cells (Figure 6C) and peritubular capillaries were dilated with swollen endothelial cells and marginated mononuclear cells, and glomeruli contained segmental mononuclear cell infiltrates that included Mac2⁺ macrophages (Figure 6D). Histopathology of F1 grafts retrieved from B6.CCR5^{-/-} recipients on day 65 post-transplant manifested severe chronic kidney graft injury with prominent interstitial fibrosis, severe glomerulopathy and arteriopathy (Figure 6E). C4d was detected diffusely in glomerular and peritubular capillaries (Figure 6F) and moderate T cell and macrophage infiltrates were present in grafts at day 65 post-transplant (Figure 6G and H).

Graft expression levels of inflammatory cytokine and fibrogenic factor mRNA

The development of marked chronic injury in F1 semi-allogeneic kidney grafts was further investigated by determining intragraft expression of genes encoding inflammatory cytokines and fibrogenic factors. On days 7, 20, and 60 post-transplant isografts, A/J allografts and F1 semi-allogeneic grafts were harvested from wild type C57BL/6 and B6.CCR5^{-/-} recipients, tissue mRNA was isolated and gene expression quantitated by qPCR (Figure 7). Similar to our previous observations (19), expression of genes encoding NK cell mediated functions, including perforin, granzyme B, IFN- γ and FasL, peaked on day 7 post-transplant in isografts and allografts and then quickly fell to near background levels. With the exception of granzyme B, expression of these genes in F1 grafts appeared at lower levels on day 7 post-transplant but increased to peak levels by day 20 and then decreased. Granzyme B was expressed at high levels at day 7 post-transplant and then decreased to background by day 20. Expression of genes encoding chemokines for macrophage recruitment (CCL2) also reached peak on day 7 post-transplant in allografts and then decreased whereas expression of genes encoding chemokines for neutrophil recruitment (CXCL1 and CXCL2) appeared on day 7 post-transplant and continued to increase to time of rejection. In F1 grafts mRNA expression of all of these genes reached peak at day 20 post-transplant and then decreased. Finally, expression of genes encoding profibrogenic factors, including MMP7, CTGF, P-selectin, E-cadherin, and N-cadherin appeared in isografts, allografts and F1 grafts on day 7 post-transplant and continued to increase in allografts until the time of rejection and in F1 grafts with time post-transplant, with the exception of MMP7, which peaked on day 20 and then decreased.

Peri-transplant NK cell depletion in B6.CCR5^{-/-} recipients of F1 semi-allogeneic kidney grafts

Since ischemia-reperfusion induced inflammation and NK cell activity was evident in F1 semi-allogeneic kidney grafts in B6.CCR5^{-/-} recipients during the first 10–12 days post-transplant the effect of peri-transplant NK cell depletion was tested on the development of the chronic graft injury. The grafts were harvested on day 65 post-transplant and tissue sections stained for histological evaluation (Figure 8A). When compared to F1 semi-allogeneic grafts from non-treated recipients where chronic injury was diffuse and most glomeruli were sclerotic (Figure 6E), peri-transplant depletion of NK cells resulted in attenuated chronic graft injury with focal infiltrates of T cells and macrophages and sclerosis in less than 50% of glomeruli. The attenuated injury in the F1 semi-allogeneic grafts from peri-transplant anti-NK1.1 mAb treated recipients was accompanied by significant decreases in the levels of mRNA encoding the profibrogenic factors P-selecting and CTGF as well as decreases in MMP7 and VCAM-1 but not in IFN- γ and perforin (Figure 8E).

DISCUSSION

NK cells have been implicated as important effector cells in antibody-mediated allograft injury. Recent studies from Hidalgo and Halloran reported the expression of NK cell associated transcripts in biopsies from kidney grafts experiencing ABMR (14, 24, 25). These NK cell associated transcripts are also expressed in kidney allografts experiencing ABMR in CCR5^{-/-} recipients and depletion of NK cells beginning at the time serum DSA reached peak titers abrogates rejection and prolongs graft survival (19). However, the activation of NK cells directly within kidney allografts during the production of DSA has not been clearly identified and was the focus of the current study. The two key findings of this study are the rapid infiltration and activation of NK cells within kidney allografts that correlate with the serum DSA titers and that in the absence of NK cell activation high DSA titers do not mediate acute graft rejection but rather initiate an indolent and progressive chronic injury that in time will lead to graft failure.

The first key finding is that as DSA titers increase in CCR5^{-/-} recipients NK cell numbers increase within the allograft. At least one mechanism underlying these increases is NK cell proliferation within the allograft. Approximately 40% of the NK cells are proliferating within the kidney allograft when examined at any single time point post-transplant, suggesting that continued recruitment of NK cells from the recipient periphery to the graft may also contribute to NK cell accumulation within the kidney allografts. The stimuli inducing NK cell proliferation during acute ABMR have not yet been identified but studies in virus infection and tumor models have identified IL-15 as a key factor promoting NK cell proliferation and viability (26–30). Proliferation of FcR γ ⁺ NK cells and activation to produce IFN- γ during 11–13 day coculture with human cytomegalovirus infected cells was dependent on the addition of seropositive IgG to the cultures, suggesting antibody-induced cross-linking of CD16 on the NK cells and/or production of NK cell activation factors by the infected cells (31). Reports in animal models have indicated that NK cell mediated xeno- or allo-graft injury requires FcR γ expression and intact DSA (32, 33). Further evidence for a role of FcR γ in antibody-mediated NK cell activation is provided by the co-expression of

transcripts in kidney graft biopsies during ABMR with those induced by cross-linking CD16a on NK cells in vitro (34). However, only a small percentage of kidney allograft infiltrating NK cells in CCR5-deficient recipients express CD107a at the time DSA reaches peak titers on day 14 post-transplant and this is further reflected by the low expression of mRNA encoding immune functions, including IFN- γ , perforin and granzyme B at that time (Figure 8 and Reference 19), raising questions about the mechanisms and timing of NK cell effector functions in mediating graft injury in the presence of high DSA titers.

Kidney allograft infiltrating NK cells were composed of populations expressing high and low levels of NK1.1. More NK1.1^{high} cells were proliferating early after transplant but by day 14 post-transplant equivalent proliferation of NK1.1^{high} and NK1.1^{low} cells was observed. This change may reflect a transition of NK1.1^{high} cells to the NK1.1^{low} cells as they are activated within the kidney allograft or the recruitment of the NK1.1^{low} cells into the allograft. NK1.1 is a member of the NKR-P1 family of NK cell activation molecules and activation through NK1.1 is reported to require an association with the CD16/Fc γ III receptor (22, 23). However, we observed that CD16 expression is not decreased on NK1.1^{low} cells in the graft, suggesting that CD16 expression is not linked to NK1.1 expression. Furthermore, both NK1.1 expression on NK1.1^{low} cells and decreased CD16 expression on NK1.1^{high} cells within kidney allografts did not result in any obvious change in NK cell activation to proliferate or express effector function at the times peak DSA titers were achieved in kidney allograft recipients. These results raise questions about the role of NK and CD16 expression on NK cells during acute ABMR.

The second key finding of this study addressed how high titers of DSA impact kidney graft injury and outcome in the absence of NK cell activation within the graft. The absence of acute ABMR in F1 semi-allogeneic grafts was associated with absence of NK cell accumulation and activation at times peak DSA titers were reached, with all grafts maintaining function beyond day 60 post-transplant. Although the grafts did not experience acute ABMR, injury and expression of genes associated with fibrogenesis were clearly evident in the semi-allogeneic kidney grafts as early as day 20 post-transplant, suggesting that the pathway to chronic injury had been initiated at early times post-transplant. The development of this chronic injury is not observed in kidney isografts implicating the DSA as an important mediator, but it is further possible that this pathway is exacerbated by NK cell activation early after vascularization of the allografts when DSA titers are low. Early NK cell activation to express genes encoding perforin, granzyme B, IFN- γ and FasL peaked on day 7 post-transplant in isografts, allografts and semi-allogeneic grafts and may induce the expression of fibrogenic genes that are not effectively down-regulated with time post-transplant in the F1 grafts as DSA titers increase. The initial NK cell infiltration and activation in kidney grafts is instigated by ischemia-reperfusion injury and as that inflammation resolves increased NK cell infiltration and activation continues in complete MHC-mismatched allografts but not isografts or F1 semi-allogeneic grafts. Many studies have indicated a role for NK cells in exacerbating ischemia-reperfusion injury following vascular clamping of kidneys and livers (35–40).

In contrast to the current findings indicating the synergistic function of DSA and NK cells in mediating acute ABMR of kidney allografts, several studies have implicated NK cells in the

development of arterial vasculopathy in heart allografts (32, 41). The general approach used was the transfer of graft donor class I MHC-reactive mAb into T and B lymphocyte-deficient mice with heterotopic heart allografts in the presence versus absence of NK cells, where NK cell absence decreased the graft vasculopathy. Using transfer of NK cells from mice with a targeted gene deletion in various effector functions to heart allograft recipients indicated that development of graft vasculopathy required NK cell IFN- γ production and either FasL or perforin expression, suggesting that IFN- γ plus cell dependent cytotoxicity mechanisms are required to mediate the chronic injury (41). There are several important differences between these approaches and the model used to investigate NK cell activation in the current studies. Obviously, the response of heart and kidney allografts to DSA and NK cell activation may be entirely different. Second, in contrast to bolus administration of donor-reactive mAb, the induced polyclonal DSA response in CCR5^{-/-} recipients of kidney allografts appears at low titers by day 7 post-transplant before rising to peak titers a week later.

Overall the results of this study demonstrate the activation of NK cells within kidney allografts that are required to mediate acute ABMR. Following reperfusion grafts are quickly infiltrated by NK cells and in complete MHC-mismatched allografts these NK cells are activated to proliferate and express effector functions that contribute to the antibody-mediated acute graft tissue injury and rejection. Following resolution of ischemia-reperfusion injury, the expression of recipient class I MHC inhibits NK cell inactivation in F1 grafts despite the presence of high titers of DSA. However, the DSA does mediate pathological changes within the allograft that are apparent by day 20 post-transplant and progresses to the indolent chronic injury that leads to fibrosis and eventual graft failure. These studies complement those providing evidence for NK cell activation in clinical kidney grafts during ABMR and suggest that strategies interfering with NK cell activation should decrease DSA mediated tissue injury and promote improved graft function and survival.

MATERIALS AND METHODS

Mice

A/J (H-2^a), C57BL/6 (B6; H-2^b), and B6.CCR5^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6.CCR5^{-/-} mice were maintained in the Lerner Research Institute Biological Resources Unit. A/J and B6 mice were crossed to generate (A/J x B6)F1 mice for use as kidney donors. Adult males (8–12 weeks of age) were used throughout this study. The Cleveland Clinic Institutional Animal Care and Use Committee approved all animal procedures.

Kidney transplantation models

Murine orthotopic kidney transplantation was performed using microsurgical methods reported by Zhang and colleagues (42). The left kidney was flushed with heparinized Ringer's solution and harvested *en bloc* with the ureter and vascular supply from donor mice. The recipient right native kidney was removed and the donor artery and vein anastomosed to the recipient abdominal aorta and inferior vena cava. Urinary reconstruction of the donor ureter to the recipient bladder was performed as previously reported (19). The remaining native left kidney was nephrectomized on day 4 post-transplant so that recipient

survival was dependent on the transplanted kidney function. Graft survival was assessed by daily observation of recipient health and rejection was suspected upon recipient signs of illness and confirmed by histopathologic analysis. The operative success rate of kidney transplantation was >85% as determined by recipient survival beyond 2 days after native left nephrectomy. Graft recipients were treated on days 1, 4, 8 and 12 post-transplant with 250 µg control rat IgG or anti-NK 1.1 (PK136) mAb (BioXCell, Lebanon, NH) i.p. to deplete NK cells.

Flow cytometric analysis of cells in digested kidney graft tissue

The harvested kidney graft was minced and digested by incubation with collagenase for 45 min at 37°C. A single cell suspension was prepared and aliquots stained with antibodies and analyzed by flow cytometry. The following fluorochrome-conjugated antibodies were used for cell surface staining: rat anti-mouse CD3e (145–2C11) and rat anti-mouse CD49b (DX5) (eBioscience, San Diego, CA), rat anti-mouse NK1.1 (PK136) (BD Bioscience, San Jose, CA), rat anti-mouse CD16 (AT154–2) (Bio-Rad, Hercules, CA) and rat anti-mouse CD107a (1D4B) (Biolegend, San Diego, CA). Analyses were performed on a LSRII (BD Bioscience) and data analyses performed using Flowjo software (TreeStar, Inc., Ashland, OR).

Proliferation of graft infiltrating NK cells was assessed by BrdU labeling in vivo and then staining cells using BrdU Flow Kits (BD Bioscience). B6.CCR5^{-/-} allograft recipients were injected with 100 µL (1mg) of BrdU solution i.p. the day before assessment. The following day, aliquots of single cell suspensions from harvested grafts were stained with fluorescent anti-BrdU antibody and multiple fluorochrome-conjugated antibodies for NK cell surface markers and analyzed by flow cytometry.

Measurement of DSA titers

Donor-reactive IgG antibody titers were assessed in recipient serum by a flow cytometry-based analysis as previously reported (15, 16). The mean channel fluorescence of each dilution of each serum sample was determined, and the dilution that returned the mean channel fluorescence to the level observed when A/J thymocytes were stained with a 1:4 dilution of normal C57BL/6 mouse serum was divided by 2 and reported as the titer.

Immunohistochemistry analysis of kidney graft tissue

Kidney grafts were harvested and fixed in acid methanol (60% methanol and 10% acetic acid). Paraffin-embedded sections (5µm) were subjected to high temperature antigen retrieval and paraffin removal in Trilogy (Cell Marque, Hot Springs, AR) in a pressure cooker. Endogenous peroxidase activity was eliminated by incubation with 0.03% H₂O₂ for 10 min. Nonspecific protein interactions were inhibited by incubation with serum-free protein block (DAKO, Carpinteria, CA). The slides were then stained using Masson's Trichrome Stain Kit (American MasterTech, Lodi, CA) and with the following primary antibodies: monoclonal rat antibody to mouse Mac2 (Cedarlane Laboratories, Burlington, NC), rabbit polyclonal anti-CD3 antibody (Abcam, Cambridge, MA), and rabbit polyclonal antiserum to mouse C4d produced as described previously (43). Staining antibodies were visualized using rat or rabbit on mouse HRP-Polymer Kits (Biocare Medical, Concord, CA)

as secondary antibodies followed by DAB and counterstained with hematoxylin. Slides were viewed under light microscopy, and images captured using ImagePro Plus (Media Cybernetics, Silver Springs, MD).

RNA extraction, cDNA synthesis, and quantitative analysis of gene expression

Total cell RNA was isolated from snap-frozen kidney graft tissue using RNeasy Mini Kits (QIAGEN, Valencia, CA) and reverse transcription performed using High-Capacity cDNA Archive Kits (Applied Biosystems, Foster City, CA) with commercially available primers. qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). For quantification of mRNA expression, data were normalized to Mrpl32 gene expression.

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Comparisons between kidney allograft survival times were analyzed using Kaplan-Meier survival curves and log-rank (Mantel Cox) statistics. Statistical differences between two experimental groups were analyzed using two-tailed *t* tests with *P* values <0.05 considered as significant. Error bars represent standard error of the mean for each experimental group.

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Figure 1 I. Identification of graft infiltrating NK cell in complete MHC-mismatched kidney allografts.

Groups of wild type C57BL/6 and B6.CCR5^{-/-} mice received kidney B6 isografts or A/J allografts. On days 7 and 14 post-transplant grafts were harvested and weighed, digested to obtain single cell suspensions and aliquots were stained with fluorochrome-labeled mAb for flow cytometry analysis. **(A)** Strategy to first gate the graft infiltrating lymphocyte population on day 14 post-transplant and then gate the CD3e⁻NK1.1⁺DX5⁺ NK cells to identify the NK1.1^{high} and NK1.1^{low} populations and their expression of CD107a. **(B)** The

flow cytometry analyses were used to determine numbers of total NK cells and NK1.1^{high} and NK1.1^{low} cells per mg of iso- and allo-graft tissue on day 7 post-transplant. **(C)** Histogram of side scatter (SSC) of gated NK1.1^{high}DX5⁺ and NK1.1^{low}DX5⁺ cells in the allografts on day 14 post-transplant was compared. **(D)** Cell suspensions from the spleen of naïve C57BL/6 mice and B6.CCR5^{-/-} allograft recipients on day 7 post-transplant were stained and analyzed by flow cytometry for detection of the NK1.1^{high}DX5⁺ and NK1.1^{low}DX5⁺ cells.

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Figure 2 I. Temporal analysis of NK cells infiltrating kidney grafts.

Groups of wild type C57BL/6 and B6.CCR5^{-/-} mice received B6 isografts, complete MHC-mismatched A/J allografts, or semi-allogeneic (A/J x B6)F1 kidney grafts (n = 6–9 grafts per time point). On days 7 and 14 post-transplant grafts were harvested and weighed, digested to obtain single cell suspensions and aliquots were stained with fluorochrome-labeled mAb for flow cytometry analysis. The flow cytometry analyses were used to determine numbers of total (A) NK cells, (B) NK1.1^{high}, (C) NK1.1^{low}, (D) CD107a⁺ NK, (E) CD107a⁺ NK1.1^{high} and (F) CD107a⁺ NK1.1^{low} cells per mg graft tissue. Numbers for each NK cell

population is indicated for individual grafts with the bar indicating the mean number per mg graft tissue. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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Figure 3 I. Proliferation of NK cells infiltrating complete MHC-mismatched A/J kidney allografts.

Groups of B6.CCR5^{-/-} mice received complete MHC-mismatched A/J allografts (n = 6 grafts per time point). BrdU was injected i.p. on day 6 or day 13 post-transplant and grafts were harvested and weighed the following day on day 7 or 14 post-transplant. Each graft was digested to obtain single cell suspensions and aliquots were stained with fluorochrome-labeled mAb for flow cytometry analysis of total NK cells and the NK1.1^{high} and NK1.1^{low} cell populations in each graft. **(A)** Representative gating of BrdU⁺NK cells in flow

cytometry analysis of allograft infiltrating NK1.1^{high} and NK1.1^{low} cell populations on days 7 and 14 post-transplant. **(B)** Absolute number of total BrdU⁺NK cells/mg graft tissue on day 7 and 14 post-transplant. **(C)** The frequency of BrdU⁺ NK1.1^{high} and NK1.1^{low} cell populations infiltrating allografts on days 7 and 14 post-transplant. **(D)** The numbers of allograft infiltrating BrdU⁺ NK1.1^{high} and NK1.1^{low} cells/mg graft tissue on days 7 and 14 post-transplant. *p<0.05, **p<0.01.

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Figure 4 I. CD16 expression on allograft infiltrating and spleen NK1.1^{high} and NK1.1^{low} cells. Single cell aliquots of graft infiltrating cells and spleen cells from B6.CCR5^{-/-} recipients of A/J allografts and spleen cells from a naïve C57BL/6 mouse were stained with antibodies and gated as in Figure 1A to analyze expression of FcγIII/CD16 on NK1.1^{high} and NK1.1^{low} cells. Results shown are representative of 4 different graft and spleen cell samples per day post-transplant.

Figure 5 I. Survival of complete MHC-mismatched and semi-allogeneic F1 kidney grafts in B6.CCR5^{-/-} recipients.

Groups of B6.CCR5^{-/-} mice received complete MHC-mismatched A/J kidney allografts (n = 6) or semi-allogeneic (A/J x B6)F1 kidney grafts (n = 5) and nephrectomy of the remaining native kidney was performed on day 4 post-transplant. (A) Survival of kidney grafts was followed by daily examination of overall animal health and confirmed by histologic analysis of harvested grafts. ****P* < 0.001 (B) At the indicated times post-transplant, serum was obtained from individual graft recipients and tested for reactivity to

graft donor thymocytes to determine the titer of donor-reactive antibody. Data indicate mean titer for each graft recipients group $\pm$ SEM. All significant differences between DSA serum titers in B6.CCR5^{-/-} recipients of A/J allografts and F1 semi-allogeneic grafts vs serum titers in wild type C57BL/6 recipients of A/J allografts are indicated by *P < 0.05 and ***P < 0.001. DSA, donor-specific antibody.

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Figure 6 I. Histological evaluation of semi-allogeneic (A/J x B6)F1 kidney grafts in B6.CCR5^{-/-} recipients.

Groups of B6.CCR5^{-/-} mice received semi-allogeneic (A/J x B6)F1 kidney grafts.

Recipients were sacrificed and the grafts harvested for histologic evaluation by staining with: (A and E) Masson's trichrome; or antibody to detect (B and F) C4d deposition, (C and G) CD3⁺ cells, and (D and H) Mac2⁺ cells.

Figure 7 I. Expression of inflammatory mediator mRNA in kidney grafts in B6.CCR5 $^{-/-}$ recipients.

Groups of wild type C57BL/6 mice received B6 kidney isografts and B6.CCR5 $^{-/-}$ mice received A/J kidney allografts or (A/J $\times$ B6)F1 semi-allogeneic kidney grafts. Grafts were harvested on the indicated day and whole cell RNA was isolated and analyzed by qPCR for expression of the indicated inflammatory mediator genes. Data indicate the mean expression levels for each group ($n=4$) on the indicated day post-transplant $\pm$ SEM. All significant differences are indicated by * $P < 0.05$ and ** $P < 0.01$.

Figure 8 I. Histological and molecular evaluation of semi-allogeneic (A/J x B6)F1 kidney grafts in B6.CCR5^{-/-} recipients given peri-transplant NK cell depleting antibody.

Groups of B6.CCR5^{-/-} mice received (A/J x B6)F1 semi-allogeneic kidney grafts and were treated with 250 mg control rat IgG or anti-NK1.1 mAb i.p. on days 1, 4, 8 and 12 post-transplant. Grafts were harvested on day 65 post-transplant and stained with: (A) Gomori trichrome; or antibody to detect (B) C4d deposition, (C) CD3⁺ cells, and (D) Mac2⁺ cells. g, normal glomerulus; Sg, sclerotic glomerulus. (E) Graft RNA was isolated from control and anti-NK1.1 treated recipients of the semi-allogeneic kidney grafts and from kidney isografts

and was analyzed by qPCR for expression of the indicated inflammatory mediator genes. Data indicate the individual and mean expression levels for each group (n =4) $\pm$ SEM. All significant differences are indicated by *P < 0.05.