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The natural killer cell activating receptor, NKG2D, is critical to antibody-dependent chronic rejection in heart transplantation

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Abstract

Chronic rejection is amongst the most pressing clinical challenges in solid organ transplantation. Interestingly, in a mouse model of heterotopic heart transplantation, antibody-dependent, NK-cell mediated chronic cardiac allograft vasculopathy occurs in some donor-recipient strain combinations, but not others. In this study, we sought to identify the mechanism underlying this unexplained phenomenon. Cardiac allografts from MHC mismatched donors were transplanted into immune-deficient C57Bl/6.rag$^{-/-}$ recipients, followed by administration of a monoclonal antibody against the donor MHC class I antigen. We found marked allograft vasculopathy in hearts from C3H donors, but near-complete protection of BALB/c allografts from injury. We found no difference in recipient NK cell phenotype or intrinsic responsiveness to activating signals between recipients of C3H versus BALB/c allografts. However, cardiac endothelial cells from C3H allografts showed an approximately two-fold higher expression of Rae-1, an activating ligand of the NK cell receptor NKG2D. Importantly, the administration of a neutralizing antibody against NKG2D abrogated the development of allograft vasculopathy in recipients of C3H allografts, even in the presence of donor specific antibodies. Therefore, the activating NK cell receptor NKG2D is necessary in this model of chronic cardiac allograft vasculopathy and strain-dependent expression of NK activating ligands correlates with the development of this disease.

1 | Introduction

Transplantation is a life-saving therapy for patients with end-stage organ failure. Although one-year allograft survival has steadily improved over the past decade,[1-5] long-term allograft survival has remained unchanged, largely due to chronic allograft rejection.[6] This poorly-understood immunologic process is an important cause of morbidity and mortality and is amongst the most pressing clinical problems in organ transplantation.

Mechanistic studies of allograft rejection have traditionally focused on the antigen-specific adaptive immune response, but the critical role of innate immunity in this process is increasingly recognized.[7-9] Natural killer (NK) cells are key components of the innate

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immune system that constitute 15-20% of circulating lymphocytes. Activated NK cells recognize and eliminate diseased cells; in addition, NK cell FCγRIII receptors recognize antibody-coated target cells, leading to antibody-dependent cell-mediated cytotoxicity.[10] Pertinent to their role in chronic rejection, NK cells interact with donor specific antibodies (DSAs) to trigger development of cardiac allograft vasculopathy (CAV) in transplanted hearts.[11] We previously reported this process to be dependent on NK cell-derived IFN-γ production and cytotoxic activity.[12] However, an unexplained finding from prior studies is that NK cell-mediated CAV occurs only in some donor-recipient strain combinations, but not in others.[13]

The current understanding of NK cell biology indicates that the transition from quiescence to activation is not driven by a single signal, but rather by an integration of signals from a diverse array of inhibitory and activating receptors.[14,15] The predominant inhibitory NK cell receptors - the killer cell immunoglobulin-like receptor (KIR) family in humans and the Ly49 family of receptors in mice - recognize 'self' class I major histocompatibility complex (MHC) molecules.[16] The predominant activating receptor on NK cells is NKG2D; ligands for this receptor are structurally similar to MHC class I molecules and include MICA / MICB and UL16 binding proteins (ULBPs) in humans, and Rae-1, H60, and MULT-1 in mice.[17] Given that NK cells are continually 'tuned' to the overall input from both activating and inhibitory receptors, we sought to test the hypothesis that strain-derived differences in NK cell receptors impact the propensity to develop antibody-dependent CAV.

2 | Materials and Methods

2.1 | Mice and in vivo procedures

C3H/HeJ (C3H, H-2^k MHC class I molecule), BALB/c-ByJ (BALB/c, H-2^d), C57Bl/6J wild-type (B6, H-2^b), and C57Bl/6.129S7-Rag1tm1Mom/J (B6.rag$^{-/-}$), mice were purchased from the Jackson Laboratory (Bar Harbor, ME). C3H males were crossed with either BALB/c or B6 females to generate F1 mice. Cardiac allografts from 10-20 weeks old donors were transplanted heterotopically into sex-matched B6.rag$^{-/-}$ recipients of the same age, as previously described.[18,19] All mice were maintained under pathogen-free conditions and were cared for according to methods approved by the American Association for the Accreditation of Laboratory Animal Care, with the approval of the Institutional Animal Care and Use Committee at the University of Colorado and the University of Florida.

Monoclonal antibodies (mAb) against H2-K^k (clone 36-7-5, BioXCell, Lebanon, NH or Southern Biotech, Birmingham, AL) or H2-K^d (clone SF1.1.10, BioXCell), were administered to transplant recipients at a dose of 30 μg in 100 μL phosphate buffered saline (PBS) twice weekly for a total of eight doses beginning the day after transplantation, as described.[11] These antibodies are non-lytic mouse anti-mouse IgG2a κ with an approximate *in vivo* half-life of 6-8 days.[11,13,20] Anti-NKG2D antibody (a blocking, non-lytic antibody[21-24]) was administered at the dose of 125 μg in 100 μL PBS (clone HMG2D, BioXCell) twice weekly for a total of eight doses beginning the day after transplantation. Clone C1.18.4 (BioXCell) was used as the IgG2a isotype control antibody for the anti-H-$2K^k$ and anti-H2-K^d antibodies; polyclonal Armenian hamster IgG (BioXCell) was used as control antibody for anti-NKG2D.

To generate polyclonal antibodies against BALB/c antigens, B6 wild-type mice were immunized (IP injection) with 30 x 10^6 splenocytes isolated from BALB/c mice and subsequently re-challenged with a repeat immunization of 30 x 10^6 BALB/c splenocytes after 30 days. Sera from the hyperimmunized B6 mice were pooled for *in vivo* use and administered to recipients as indicated at a dose of 200 μL undilute sera on post-operative day one, followed by 200 μL of a 1:5 dilution twice weekly for a total of eight doses.

2.2 | Histological Techniques and Morphometric Analysis

Allografts were removed on day 30, cross-sectioned into three parts, fixed in 10% formalin, and sections stained using Verhoeff's method[25] for morphometric analysis of coronary arteries. Briefly, images of all vessels >85 μm in diameter were captured digitally (Nikon Eclipse Ts2R microscope, Nikon NIS-Elements software, Version 5.21) at 10x magnification. Image processing and analysis with ImageJ software (NIH) was used to manually demarcate the borders of the lumen and the intima of the artery. The software then quantified the luminal and intimal areas and the neointimal index (NI) was calculated as [neointimal area / (neointimal area + luminal area)] x 100, as described.[26] This quantity was calculated for each vessel with the NI reported for each recipient representing the average of the individual values over the three cross-sections obtained per recipient.

2.3 | Recipient NK Cell Isolation and Stimulation Assay

To analyze the responsiveness of splenic NK cells *ex vivo*, 96-well high-binding flat-bottom plates (ThermoFisher (Waltham, MA)) were coated overnight with the following antibodies: control IgG 10 μg/mL, BioXCell); anti-NKG2D (clone CX5, BioLegend, San Diego, CA) 5 μg/mL; and anti-NKp46 (clone 29A1.4, BioLegend) 5 μg/mL. Plates then washed three times with PBS prior to stimulation. Single cell suspensions were generated from recipient splenic tissue of mice that had undergone transplantation, depleted of erythrocytes with red blood cell lysis buffer (Sigma-Aldrich), and cultured in the coated plates for five hours in the presence of monensin (2 uM) (Invitrogen (Carlsbad, CA)) and fluorophore (FITC)-conjugated anti-CD107a (0.5 g/mL) (Clone 1D4B, BioLegend). After stimulation, cells were stained with CD3 and NK1.1 to identify NK cells, followed by flow cytometric analysis.

2.4 | Cardiac Endothelial Cell Identification

Non-transplanted hearts and explanted allografts were recovered and a single cell suspension obtained by use of the Multi-Tissue Dissociation Kit 2 followed by use of the Debris Removal Kit per the manufacturer's instructions (Miltenyi Biotec, Germany). After isolation, cells were resuspended and processed for flow cytometric analysis. To test anti-donor MHC class I antibody binding, cardiac single-cell suspension from BALB/c or C3H mice were incubated with 0.3 mg/mL of monoclonal donor-specific antibody against H2-K^k (C3H cells) or H2-K^d (BALB/c cells) for 30 minutes at 4°C. After incubation, cells were washed and analyzed via flow cytometry.

2.5 | Flow Cytometry

Briefly, splenic NK cells were stained with CD3-PE-Cy7 (clone 145-C211, BD Biosciences, Franklin Lakes, NJ), NK1.1-PerCP-Cy5.5 (clone PK136, BD Biosciences), Ly49A-PE

(clone A1, BD Biosciences), Ly49C/I-FITC (clone 5E6, BD Biosciences), Ly49D-BV510 (clone 4E5, BD Biosciences), and Ly49G2-APC (clone 4D11, BD Biosciences) to assess Ly49 receptor expression. To detect NKG2D expression, a separate cell preparation was stained with CD3, NK1.1, and NKG2D-APC (clone CX5, BioLegend). After isolation post-transplantation, allograft endothelial cells were stained with CD45-PerCP-Cy5.5 (clone 104, BD Biosciences), CD31-FITC (clone 390, Invitrogen), Rae-1-BV421 (clone 186107, BD Biosciences), H60-PE (clone 205326, R&D Systems (Minneapolis, MN)), and MULT-1-APC (clone 237104, R&D systems). To detect anti-donor MHC Class I antibody bonding, naïve endothelial cells were stained with CD45-PerCP-Cy5.5 (clone 104, BD Biosciences), CD31-PE (clone 390, BioLegend), and mouse IgG-FITC (clone poly 4060, BioLegend) after incubation with the appropriate monoclonal antibody. After staining, cells were washed twice with PBS with 1% bovine serum albumin (BSA) and 0.1% sodium azide; data was collected using a BD LSR II or BD Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo software (BD Biosciences, Version 10.6.2).

2.6 | Statistical Methods

Summary data of neointimal index and cell population numbers were expressed as median +/− interquartile range (IQR). Mann-Whitney U test was used to compare two groups and Dunn's multiple comparison was used to compare multiple groups (non-parametric one-way analysis of variance (ANOVA). GraphPad Prism (Version 8.4.2, La Jolla, CA) was used for statistical analysis; p values < 0.05 were considered significant.

3. | Results

3.1 | Development of cardiac allograft vasculopathy is determined by the donor allograft strain

We used an established model of NK cell-dependent antibody mediated CAV in which MHC mismatched allografts were transplanted into immune-deficient B6.rag$^{-/-}$ recipients and subsequently administered monoclonal antibody against donor MHC class I antigen [11,12] (Figure 1A). In B6.rag$^{-/-}$ recipients bearing C3H (H-2^{k}) allografts, administration of anti-H2K^{k} monoclonal antibody resulted in pronounced CAV at 30 days after transplantation (NI 16.08 ± 13.10%) (Figure 1B-C). In striking contrast, recipients bearing BALB/c (H-2^{d}) allografts treated with anti-H2K^{d} monoclonal antibody did not develop CAV (NI 8.63 ± 6.20%), despite identical recipient strain, antibody isotype, equivalent DSA binding (Supplemental Figure 1) and full allogeneic donor / recipient mismatch. Use of a polyclonal anti-BALB/c serum also did not result in CAV (NI 2.65% ± 1.24%) (Figure 1C). Allografts from both strains showed little CAV when recipients were not treated with anti-MHC class I antibodies (BALB/c NI 4.92 ± 4.00%, C3H NI 3.32 ± 1.97%) or isotype control antibody (BALB/c NI 2.11 ± 10.72%, C3H NI 1.02 ± 1.36%) (Figure 1C).

To determine whether donor resistance versus vulnerability to CAV was dominant or recessive, we crossed C3H males and BALB/c females and used the resulting F1 offspring, which were heterozygous and expressed both H2K^{k} and H2K^{d} MHC class I alloantigens, as donors. As expected, untreated B6.rag$^{-/-}$ recipients bearing C3H-BALB/c F1 allografts did not develop significant CAV (NI 12.23 ± 5.45%). However, despite the presence of

one allele from the C3H genetic background, administration of C3H-specific monoclonal antibody did not result in significant CAV (NI 5.33 ± 8.66%) (Figure 1C). We concluded that NK cells and DSA are necessary, but not sufficient, for the development of chronic allograft vasculopathy and that protection of BALB/c allografts to this form of chronic vasculopathy is gene-dose dependent.

3.2 | Strain-dependent cardiac allograft vasculopathy is not mediated by Ly49 NK cell receptors

NK cells express MHC class I-specific receptors that, when engaged by their cognate ligands, inhibit activation and prevent the killing of target cells. In mice, these receptors include the lectin-like Ly49 dimers, a polymorphic family of receptors that is functionally similar to human killer cell immunoglobulin-like receptors.[16] By interacting with MHC class I molecules, which are constitutively expressed on healthy cells, these receptors mediate NK cell tolerance to 'self' but permit cytotoxicity towards cells lacking 'self' MHC expression. In B6 mice, the predominant Ly49 inhibitory receptors expressed by NK cells are Ly49C/I (which recognizes H-2^b), with other subsets expressing Ly49A and Ly49G2.[27] We began by assessing whether the lower rate of CAV in BALB/c allografts was attributable to higher expression of Ly49 inhibitory receptors by recipient NK cells. The frequency of recipient NK cells expressing individual inhibitory Ly49C/I, Ly49A, or Ly49G2 receptors after transplantation were not significantly different between recipients of BALB/c or C3H allografts (Figure 2B). We did find the surface expression of Ly49G2 in recipients (as measured by median fluorescence intensity (MFI)) of BALB/c allograft to be lower than non-transplanted B6.rag$^{-/-}$ recipients, but the expression of Ly49G2 in recipients of C3H allografts did not differ from this group or control animals (Figure 2C), suggesting that the expression of this ligand does not explain the strain-dependent differences in CAV.

Complementary to their role in promoting recognition of 'self,' Ly49 receptor expression also dictates NK cell licensing, a process by which NK cell attain their full cytotoxic functions when their self-specific Ly49 inhibitory receptors interact with self-MHC class I[16,28]. NK cell licensing is not static and NK cells adapt their responsiveness in a new MHC environment[29]. We reasoned that transplantation introduces localized expression of a 'non-self' MHC (through the allograft), and thus has the potential to alter the licensing of a recipient's NK cells. We therefore quantified the licensing of the recipient NK cells by comparing the expression of inhibitory Ly49 receptors to those of non-transplanted mice. In B6 mice, NK cells expressing Ly49C and/or Ly49I are licensed, while those that express Ly49A or Ly49G2 (which bind poorly to H-2^b) are unlicensed.[28,30] Consistent with prior studies,[31,32] we found approximately half of the recipient's NK cells to be 'unlicensed' with respect to self-MHC expression and did not express Ly49C/I, but this proportion was not significantly different from non-transplanted B6.rag$^{-/-}$ recipients (Supplemental Figure 2). This data suggests that recipient NK cell licensing is unaffected by the presence of an allograft.

In addition to inhibitory receptors, the Ly49 family also includes activating receptors, which recognize MHC or MHC-like ligands on 'altered self' cells.[16] B6 mice express the activating receptors Ly49D and Ly49H. Ly49D recognizes H-$2D^d$,[33] an MHC haplotype expressed

BALB/c cells (Table 1); it is not known to interact with H-2^k MHC class I molecules found on the C3H donor.[33,34] As the Ly49D:H-$2D^d$ interaction has been shown to be important in bone marrow rejection[35-37] and B6.rag$^{-/-}$ mice possess a significant subset of Ly49D+ NK cells, we investigated whether this subpopulation was altered in transplanted recipients. The frequency of Ly49D+ NK cells in transplanted recipients did not differ from non-transplanted animals, indicating that the ligand expression on the allograft did not affect the number of cells expressing the receptor (Figure 2B). However, we reasoned that chronic ligand exposure has the potential to lead to receptor downregulation, as the surface expression of Ly49D was lower in BALB/c allograft recipients as compared to C3H allograft recipients or non-transplanted controls (Figure 2C). In addition, closer examination of the Ly49D+ subset demonstrated that ~90% of these NK cells in transplanted recipients also co-expressed inhibitory Ly49 receptors (Figure 3). The lack of CAV development in recipients of a BALB/c allograft suggests that, although this activating receptor is present on a notable proportion of recipient NK cells, concurrent signals from inhibitory Ly49 receptors potentially negates a response. Thus, we hypothesized that the development of CAV requires additional NK cell activating signals.

A key issue underlying the interpretation of these experiments is whether or not recipient NK cells are equally responsive to stimulation. Thus, we next compared the functional responsiveness of recipient NK cells recovered from mice that had received either a C3H or BALB/c allografts. We have previously shown that CAV development requires NK cell activation and degranulation.[12] Stimulation of NK cells isolated from recipients engrafted with BALB/c or C3H allografts showed equivalent degranulation in response to stimulation by plate-bound activating antibodies, as quantified by surface expression of CD107a (Figure 4). In addition, neither group differed significantly from control NK cells from non-transplanted B6.rag$^{-/-}$ mice. Taken together, we concluded that the donor strain-related difference in rate of allograft vasculopathy is not explained by a more activated NK cell phenotype.

3.3 | Allograft expression of NKG2D activating ligands correlates with strain-related differences in the development of cardiac allograft vasculopathy

We next assessed whether the expression of the predominant NK cell activating receptor, NKG2D, explains the strain-dependent differences in chronic allograft vasculopathy. NKG2D is the dominant activating receptor on NK cells whose ligands are a diverse array of distant homologues of MHC class I molecules, including the Rae-1 family of glycoproteins, UL16-binding protein-like transcript 1 (MULT-1), and H60 glycoproteins.[38] In comparing the expression of this receptor on recipient NK cells 30 days after transplantation, we found no significant difference in the proportion of NK cells expressing NKG2D, the absolute number of splenic NK cells expressing NKG2D, or mean fluorescence intensity of NKG2D expression on NK cells derived from recipients that had received a BALB/c versus C3H allograft (Figure 5A-D), suggesting that NK cells from both groups of recipients can respond equally to NKG2D receptor activation.

To determine whether higher expression of NKG2D ligands on C3H allografts mediates chronic allograft vasculopathy, we next assessed the expression of these ligands on allograft

CD45-CD31+ endothelial cells isolated 30 days after transplantation. We found comparable low-level expression of the NKG2D ligands H60 and MULT1 in both BALB/c and C3H allografts (Supplemental Figure 3), but markedly higher expression of Rae-1 in C3H, as compared to BALB/c, allografts ($p = 0.0082$, Figure 6A). Examination of pre-transplant Rae-1 expression on cardiac endothelial cells derived from C3H mice demonstrated a similarly higher level of expression in comparison to endothelial cells derived from BALB/c hearts (Figure 6A). We had earlier demonstrated that the predisposition of C3H hearts to allograft vasculopathy displayed a recessive pattern of inheritance (Figure 1C); we thus sought to determine whether this effect was related to the expression of NKG2D ligands in F1 C3H allografts. To assess this, we crossed C3H males and B6 females and used the resulting F1 offspring as allograft donors. Interestingly, B6.rag−/− recipients bearing C3H-B6 F1 allografts that received DSA (against H-2K^k) did not develop significant cardiac allograft vasculopathy (NI 6.80 ± 1.46%) (Figure 6C) and C3H-B6 F1 animals possessed a low percentage of Rae-1+ endothelial cells as compared to wild-type C3H wild-type mice ($p = 0.0028$). In addition, C3H-BALB/c F1 animals also demonstrated a low percentage of Rae-1+ endothelial cells comparable to those isolated from BALB/c mice pre- and post-transplant (Figure 6A); these results are consistent with the hypothesis that the levels of donor expressed NKG2D ligand impacts the propensity to develop allograft vasculopathy.

Lastly, to determine whether NKG2D receptors were required to trigger allograft vasculopathy, we assessed the effect of an NKG2D blocking antibody in the development of CAV. As shown before, recipients of C3H allografts developed CAV when administered DSA (Figure 1C). Strikingly, concurrent administration of anti-NKG2D was sufficient to abrogate antibody-dependent CAV in these allografts (NI 5.75 +/− 4.62%) (Figure 6B). Taken together, these data indicate that the enhanced NK cell mediated CAV observed in C3H allografts is attributable to increased expression of the NKG2D ligand Rae-1 by C3H cardiac endothelial cells.

4 | Discussion

The role of NK cells in solid organ transplantation is context-dependent: NK cells are effector cells in acute rejection[39] and chronic antibody-mediated rejection[11,12] but also promote allograft survival by inhibiting the alloimmune response initiated by donor antigen presenting cells.[40-43] We add to this literature by showing that the function of NK cells in antibody-mediated chronic rejection is regulated by a balance between negative signals from inhibitory receptors for MHC class I and positive signals from NKG2D and DSA.

The “rheostat” model of NK cell responsiveness suggests that these cells are continually tuned by the inhibitory and stimulatory interactions encountered in a given environment, allowing NK cells to be “self-tolerant” but also respond to changes in normal cells.[44] A key family of receptors contributing to this balance is the Ly49 family of inhibitor receptors, which engage MHC I molecules at steady state. The ability of an NK cell subset to lyse a particular allogeneic target is determined by the class I specificity of the two or three Ly49 receptors expressed by most NK cells [45]. Interestingly, inhibitory Ly49 receptors display ligand ‘promiscuity’ and can engage several MHC class I ligands (Table 1) beyond those classified as ‘self.’ In skin and solid organ transplant models, NK cells are not sufficient to

induce allograft injury.[11,12,46-48] This was re-demonstrated in the current work and may be explained by the recognition of non-B6 MHC haplotypes by B6 NK cells with generation of a sufficient inhibitory signal to prevent activation. In addition, the interaction between different MHC class I ligands and Ly49 receptors result in different degrees of NK cell inhibition. For instance, studies in bone marrow transplantation have suggested that there is a 'broader' inhibitory signal provided by H-2^d, thereby a 'stronger' activation signal is required to overcome this and initiate NK cell-mediated rejection.[37] This is pertinent to our study, as B6.rag$^{-/-}$ recipients were shown to be more 'tolerant' to BALB/c (H-2^d) allografts. Taken together, this suggests that 'uninhibited' NK cells are not driving the response against allograft; rather, concurrent activating signals from the donor may be necessary or rate-limiting in triggering damage in order to overcome the dominant inhibition exerted by the Ly49 inhibitory receptors.

NKG2D is an activating receptor of both human and murine cytotoxic lymphocytes including NK cells.[49] NKG2D ligands are expressed at a low level on healthy cells and are upregulated by heat, oxidative stress, infection, or malignant transformation.[38,50] Expression is also influenced by genetic background[37,51] and constitutive expression of NKG2D ligands have been described in the gut epithelium, airway epithelium, hepatocytes, and myocytes.[37,50,52] Pre-clinical studies have shown that neutralization of NKG2D results in improved allograft survival in cardiac and tracheal transplant models.[39,53,54] It has also been shown that the adaptive alloimmune response can drive NKG2D ligand expression,[39] rendering an allograft prone to NK cell-mediated injury. Conversely, another study reported that NKG2D blockade in recipients resulted in early allograft infiltration of NK cells, as well as enhanced frequencies of dendritic cells and decreased regulatory T cells. Significantly, NKG2D blockade in combination with co-stimulation blockade resulted in significantly reduced allograft survival, and thus the authors argued for a regulatory role for these cells in maintaining transplant tolerance.[55] It is difficult to directly compare our results to those prior given differences in our model system used. The interaction of NKG2D and its ligands, specifically Rae-1, was of importance in the development of CAV in our model as there was a clear difference in ligand expression between the two donor strains utilized and NKG2D receptor blockade abrogated the development of CAV. We do recognize that this does not preclude other potential effects of NKG2D, such as on NK cell trafficking to the allograft, or of other NK cell activation signals. We also recognize other genetic differences exist between BALB/c and C3H mice and that the observed effect described was only studied in specific donors / recipients and may not apply to other strain combinations. Lastly, unlike comparable studies in both transplantation and tumor immunology[38,53,56] continued expression of Rae-1 by cardiac endothelial cells was not sufficient to result in appreciable NKG2D receptor downregulation or reduced recipient NK cell responsiveness in our model. In view of the varied studies indicating disparate roles in NKG2D in transplant outcomes, our study demonstrated a clear role for this receptor in mediating chronic allograft injury in a model of NK cell and antibody-mediated rejection.

We recognize several limitations in our study: as key cells of the innate immune system, NK cells respond to a number of inflammatory signals; it is plausible that ischemia-reperfusion injury and cellular stress that occurs at the time of allograft implantation could induce expression of several NKG2D ligands and contribute to early allograft injury; we only

queried ‘late’ injury in this process and focused on the ligand that demonstrated sustained expression over time. In our model, only a single monoclonal anti-donor Class I antibody was utilized per donor mouse strain; clinically, multiple class I DSAs can be present in a given recipient. Lastly, our work was focused on NK cell-mediated mechanisms of CAV in a reductive model that intentionally eliminated the contribution of the adaptive immune response, specifically T cells. Since NK cells can both inhibit and enhance T cell responses, and cytotoxic CD8+ T cells express NKG2D[57,58] the role of NKG2D ligands in human CAV is likely to be significantly more complex.

In conclusion, our study provides a mechanistic explanation for the development of CAV only in specific instances, since in many donor / recipient strain combinations, the allograft donor will possess MHC haplotypes that react with the majority of inhibitory Ly49 receptors expressed by NK cells of the recipient and no injury will occur. Our results indicate that CAV only occurs when another activating signal (in this case, NKG2D) or a synergistic combination of signals (addition of DSA) overcomes this specific inhibition. Within the context of solid organ transplantation, this reflects the importance of the role of NK cells in the development of cardiac allograft vasculopathy, as well as the balance of signals required to result in their activation. This work has several implications for future research: in light of our findings, the effect of blocking NK cell activating receptors in transplant recipients on NK cell effector functions and NK interactions with other immune cells should be determined. Our study may also inform whether recipients with high NK cell ligand expression (for example, due to ischemia-reperfusion injury or infection) or those with allograft donors expressing specific NKG2D ligand(s) benefit from NK cell-targeted therapy, as a new therapeutic strategy for the prevention of long-term allograft dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
CAV	cardiac allograft vasculopathy
DSA	donor specific antibody
IFN-γ	interferon gamma
IQR	interquartile range
NK	natural killer

NI	neointimal index
NKG2D	natural killer group 2D
MHC	major histocompatibility complex

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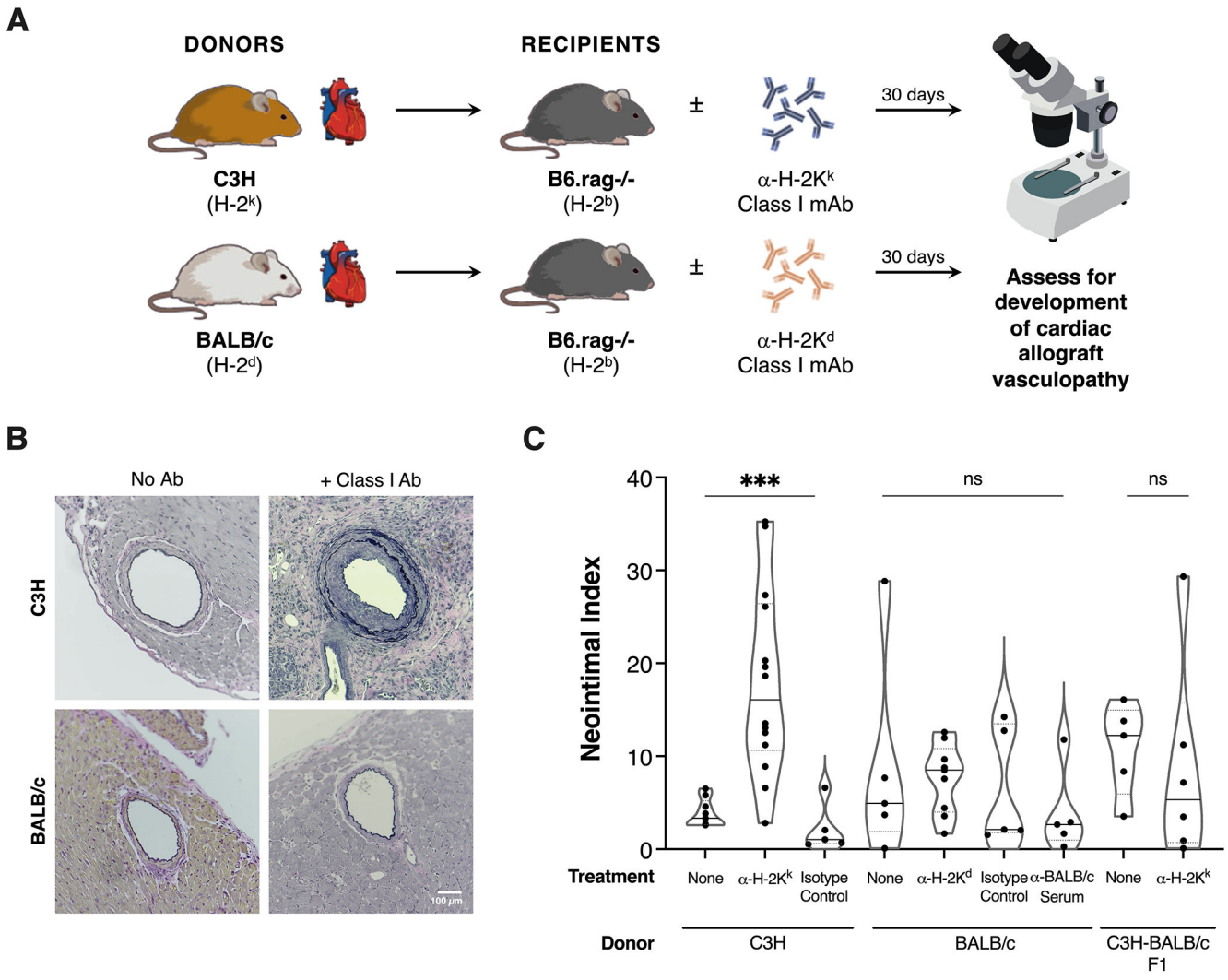

Figure 1. The development of donor specific antibody-mediated coronary allograft vasculopathy is dependent on donor strain.

(A) Schematic representation of transplant donor / recipient combinations and their MHC class I haplotypes. (B) Verhoff's elastin stain of representative coronary arteries from B6.rag$^{-/-}$ recipients that received either a C3H or BALB/c donor allograft with or without administration of donor specific antibody (α-H-2K^{k} or α-H-2K^{d} IgG2a). Scale bar in the last panel equivalent in all other images; original magnification 100X. (C) Donor allografts from the indicated strains were transplanted into B6.rag$^{-/-}$ recipients and received either no treatment or were administered either monoclonal Class I donor specific antibody (α-H-2K^{k} or α-H-2K^{d} IgG2a), polyclonal anti-BALB/c serum, or isotype control antibody. Each data point represents one recipient mouse; solid and dotted lines represent median and inter-quartile range (IQR), respectively (ns, no significant difference; ***p ≤ 0.001).

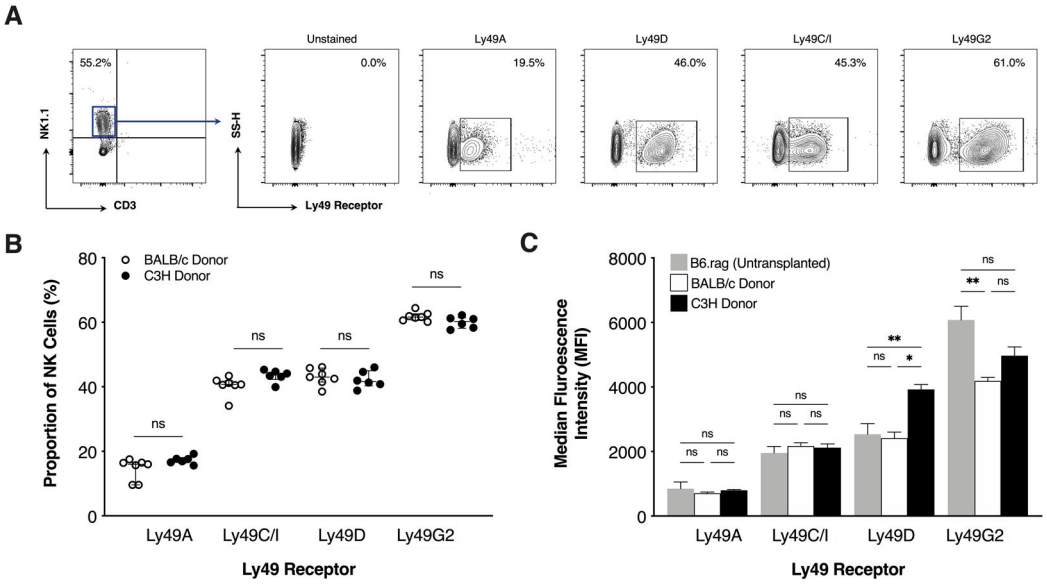

Figure 2. Ly49 receptor expression on recipient NK cells is not significantly altered by donor allograft strain.

Splenic NK cells from B6.rag$^{-/-}$ recipients that received either a BALB/c or C3H cardiac allograft were obtained 30 days after transplantation and stained for their Ly49 receptor repertoire. (A) Representative flow cytometry plots of the indicated markers expressed by freshly isolated splenic NK cells. Cells were initially gated on single-cell lymphocytes by light scatter characteristics, followed by staining for CD3, NK1.1, and the individual Ly49 receptors. Fluorescence minus-one (FMO) samples were used to determine the gating strategy. (B) Percentage of NK cells (CD3− NK1.1+) of the indicated Ly49 receptor. Each data point represents one recipient mouse; horizontal line and error bars represent median and IQR. (C) Median fluorescence intensity (MFI) of recipient NK cells expressing the indicated Ly49 receptor based on allograft donor. Data is represented as median ± IQR (ns, no significant difference, *p < 0.05; **p <0.01).

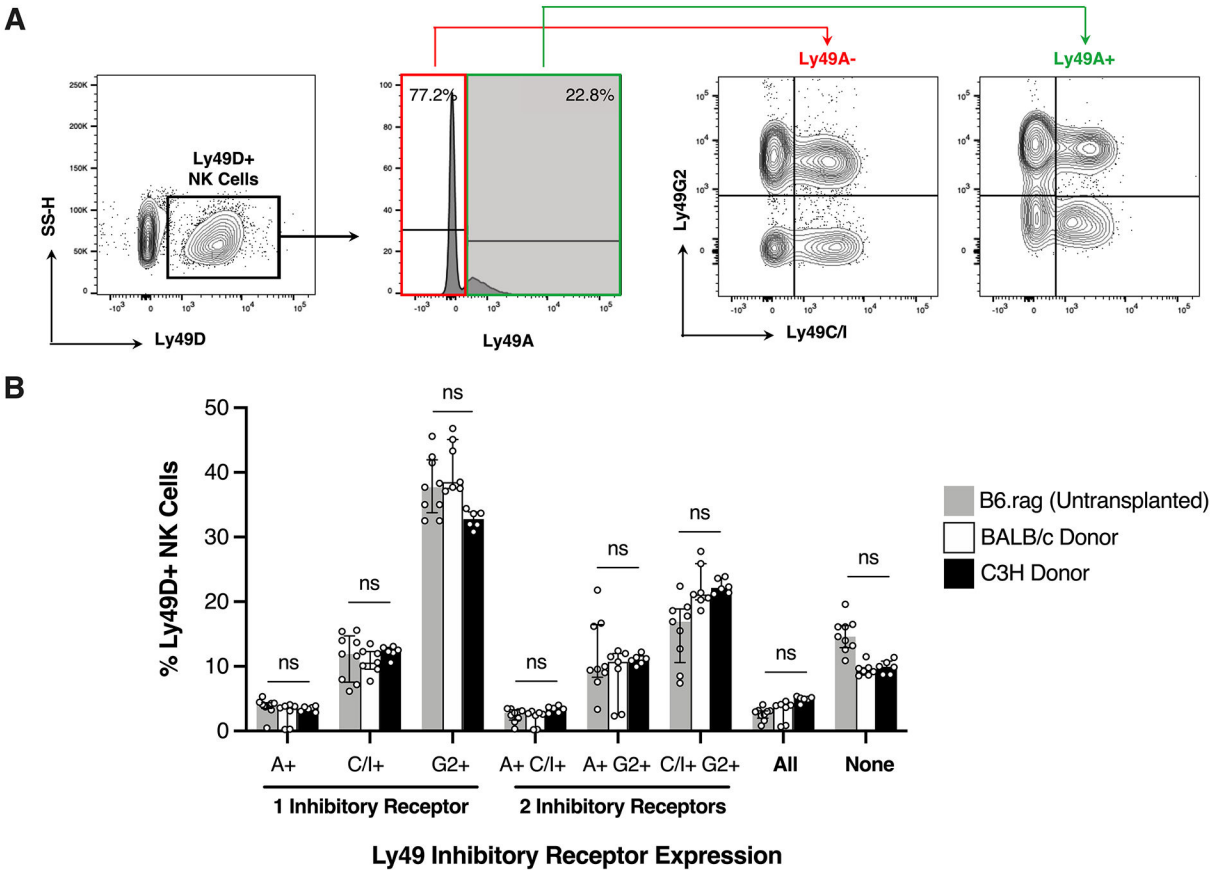

Figure 3. The majority of Ly49D+ NK cells co-express one or more inhibitory Ly49 receptors.

(A) Representative gating strategy to determine Ly49 inhibitory receptor co-expression on Ly49D+ NK cells. Cells were gated on CD3− NK1.1+ Ly49D+ as shown in Figure 2A. Of the Ly49D+ subset, cells were subsequently divided based Ly49A+ expression; both subsets were analyzed for the presence of co-expression of Ly49C/I and Ly49G2. Fluorescence minus-one (FMO) samples were used to determine the gating strategy. (B) Percentages of Ly49D+ NK cells expressing one, two, all (3), or none (0) inhibitory Ly49 receptors were derived from the flow cytometry data indicating that the majority of recipient Ly49D+ cells co-expressed inhibitory Ly49 receptors. Data is expressed as median ± IQR with each data point representing one recipient animal (ns, no significant difference).

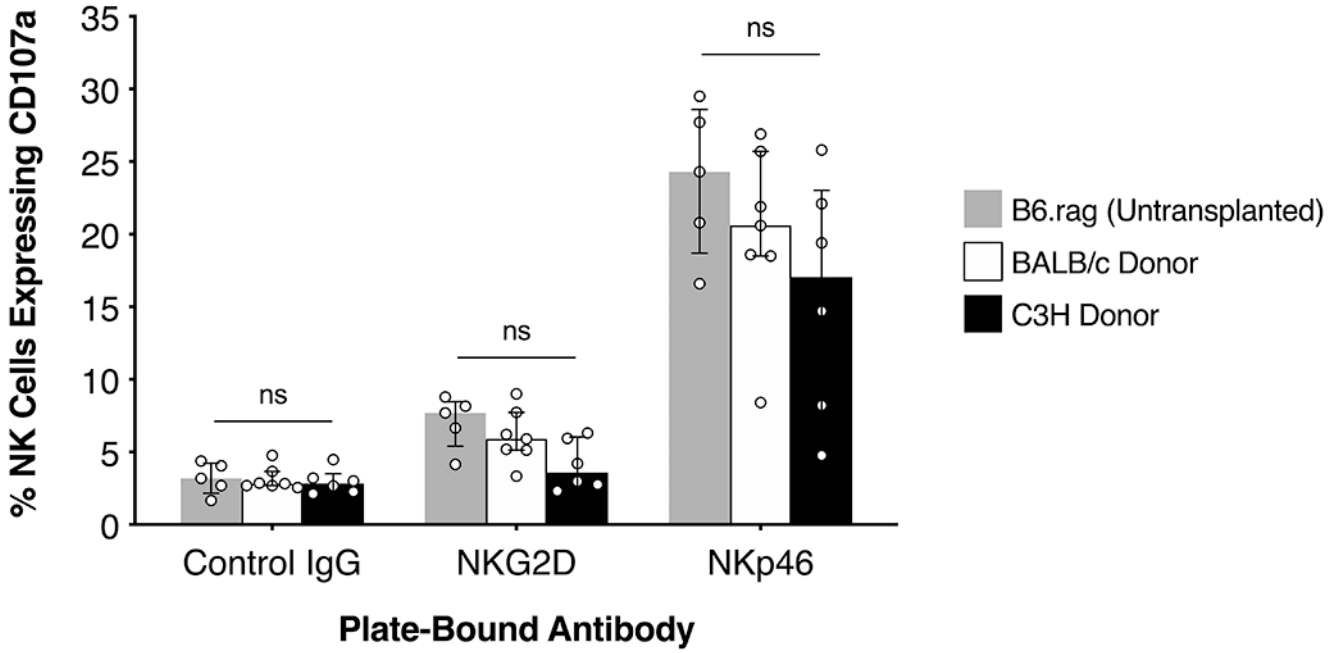

Figure 4. Recipient NK cell activation is unchanged in recipients of different allograft donors.

Splenic NK cells from B6.rag$^{-/-}$ recipients that received either a BALB/c or C3H cardiac allograft were obtained 30 days post-transplantation and stimulated with plate-bound antibodies in the presence of CD107a. NK cells isolated from non-transplanted B6.rag$^{-/-}$ mice were used as the control. Data is expressed as median ± IQR with each data point representing one recipient animal (ns, no significant difference.

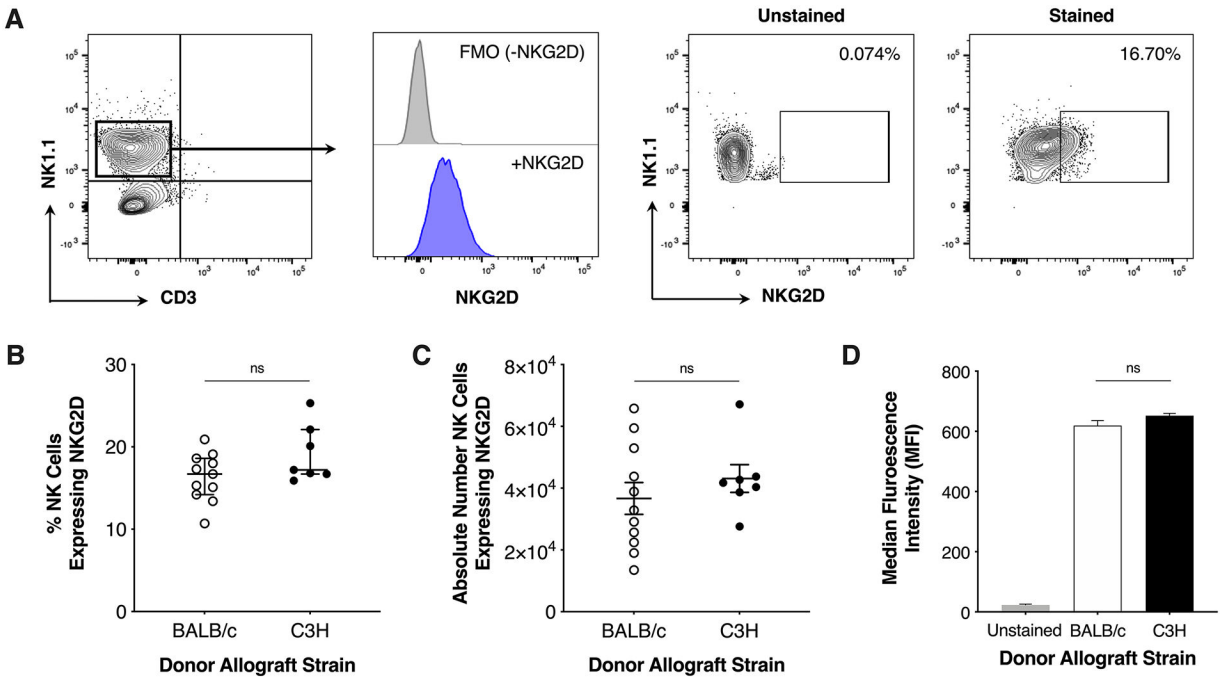

Figure 5. Recipient NK cell NKG2D receptor expression is not altered by donor strain.

Splenic NK cells from B6.rag$^{-/-}$ recipients that received either a C3H or BALB/c cardiac allograft were obtained 30 days post-transplantation and stained for the presence of the NKG2D receptor. (A) Representative flow cytometry plots of the indicated markers expressed by freshly isolated splenic NK cells. Cells were initially gated on single-cell lymphocytes (FSC vs SSC), followed by staining for CD3, NK1.1, and NKG2D. Fluorescence minus-one (FMO) samples were used to determine the gating strategy. With respect to the donor allograft strain, no significant difference was noted in the (B) percentage of recipient NK cells expressing NKG2D, (C) absolute number of recipient NK cells expressing NKG2D, or the (D) the median fluorescence intensity (MFI) of recipient NK cells expressing the receptor. Data is expressed in panels B-C as median ± IQR (ns, no significant difference).

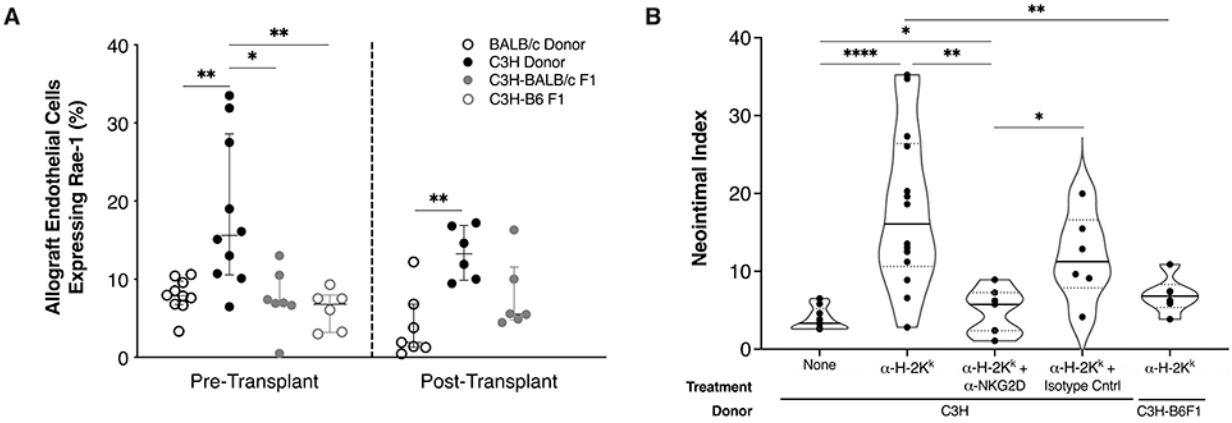

Figure 6. NKG2D ligand expression correlates with strain differences in CAV development.

(A) Cardiac endothelial cells were isolated from naïive donor mice or at 30-days post-transplant and analyzed for expression of Rae-1. (B) Donor allografts from the indicated strains were transplanted into B6.rag$^{-/-}$ recipients and received either no treatment or were administered class I donor specific antibody (α-H-2K^{k}), or donor specific antibody in conjunction with α-NKG2D or polyclonal Armenian hamster isotype control antibody. Data is expressed in panels A-B as median ± IQR (ns, no significant difference, *$p < 0.05$. **$p \leq 0.01$, and ****$p \leq 0.0001$).

Table 1

Class I specificity of Ly49 Receptors [27,30,31,60]

Receptor	Class I Ligand	Function
Ly49A	H-2^{d}, H-2^{k}	Inhibitory
Ly49C/I	H-2K^{b}, H2^{d,k,s}	Inhibitory
Ly49G2	H-2D^{d}, H2^{k}	Inhibitory
Ly49D	H-2D^{d}	Activating
Ly49H	N/A (m157)	Activating