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# NK Cells Can Trigger Allograft Vasculopathy: The Role of Hybrid Resistance in Solid Organ Allografts<sup>1</sup>

Shuichiro Uehara,\* Catharine M. Chase,\* William H. Kitchens,<sup>2\*</sup> Harris S. Rose,<sup>2\*</sup> Robert B. Colvin,<sup>†</sup> Paul S. Russell,\* and Joren C. Madsen<sup>3\*</sup>

Progressive arterial stenosis (cardiac allograft vasculopathy (CAV)) is a leading cause of long-term failure of organ transplants. CAV remains intractable, in part because its mechanisms are insufficiently understood. A central proposition is that MHC-driven alloimmune processes play a necessary role in CAV, as shown by the absolute requirement for histoincompatibility between donor and recipient for its production. Two immunological pathways have been implicated involving reactivity to donor MHC Ags by either T or B cells. In this study, we use a novel system of semiallogeneic cardiac transplants between parental donors and F1 hybrid recipients to provide evidence that NK cells, members of the innate immune system, also contribute to the generation of CAV in mice. This finding marks the first demonstration that the hybrid resistance phenomenon occurs in solid organ allografts. Extension of these experiments to recipients deficient in T cells demonstrates that this third pathway of CAV, the NK cell-triggered pathway, involves the recruitment of T cells not responsive to donor alloantigens. Finally, transplants performed with donors or recipients deficient in IFN- $\gamma$  revealed that recipient-derived IFN- $\gamma$  is necessary for CAV formation in parental to F1 transplants, suggesting a possible effector mechanism by which NK cells can promote CAV. Together, these results define a previously unknown pathway toward CAV and assign a novel role to NK cells in organ allograft rejection. *The Journal of Immunology*, 2005, 175: 3424–3430.

Cardiac allograft vasculopathy (CAV)<sup>4</sup> is characterized by concentric proliferation of neointimal cells in the allograft arteries, leading to a progressive and diffuse arteriosclerosis that often culminates in luminal occlusion, ischemic injury, replacement fibrosis of myocardium, and eventual allograft failure (1). Angiographic studies demonstrate CAV in almost 50% of cardiac allografts 5 years after transplant, although more sensitive intravascular ultrasound studies detect intimal thickening in >80% of allograft recipients (2, 3). This ubiquity of CAV explains why it is the leading source of posttransplant mortality after the first year, leading to the death or retransplant of two of every three patients with severe CAV (2, 4). Development of effective therapies for CAV has been hindered by an incomplete understanding of many basic aspects of its pathophysiology, including an insufficient grasp of which cell populations are responsible for promoting CAV formation.

The contribution of T and B cells to the development of CAV is well described (5–7). However, recent studies suggest that the adaptive immune system is not always solely responsible for CAV

formation, because mice with T and B cells rendered fully unresponsive to donor Ags through the induction of neonatal tolerance or mixed chimerism still developed CAV (8). In search of potential contributions by innate immunity to CAV development, we have focused on the role of NK cells, a subset of lymphocytes that classically has not been implicated in the alloresponse to solid organs (9). To isolate NK cell activity directed against a heart allograft from T and B cell alloreactivity, we transplanted parental hearts into F1 hybrid mice. This strategy was suggested from previous studies showing that bone marrow transplanted from parental strains of mice to F1 hybrids between the parental strain and a second strain is rejected (10). This phenomenon, first observed with transplantable lymphomas by Snell (11) and subsequently termed “hybrid resistance” (10), can be attributed solely to the action of NK cells and their ability to both recognize and react to the absence of self MHC class I products on the surfaces of target cells, the “missing self” hypothesis (12–14). The parent to F1 system provides a reliable and uncontrived means of eliminating ordinary host anti-donor T and B cell responses while maintaining normal host NK responses to organ allografts.

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<sup>4</sup> Abbreviations used in this paper: CAV, cardiac allograft vasculopathy; CB6F1, (C57BL/6  $\times$  BALB/c)F<sub>1</sub>.

## Materials and Methods

### Mice

C57BL/6 (H-2<sup>b</sup>), BALB/c (H-2<sup>d</sup>), (C57BL/6  $\times$  BALB/c)F<sub>1</sub> (H-2<sup>bxd</sup>) (CB6F1), C3H/HeJ (H-2<sup>k</sup>), (C57BL/6  $\times$  C3H/HeJ)F<sub>1</sub> (H-2<sup>bxk</sup>), B10.D2 (H-2<sup>d</sup>), B6.129S7-Rag1<sup>tm1Mom</sup> (C57BL/6.RAG1<sup>-/-</sup>), C.129S7(B6)-Rag1<sup>tm1Mom</sup> (BALB/c.RAG1<sup>-/-</sup>), C57BL/6-*Irfng*<sup>tm1Ts</sup> (C57BL/6.GKO, H-2<sup>b</sup>), BALB/c-*Irfng*<sup>tm1Ts</sup> (BALB/c.GKO, H-2<sup>d</sup>), and C57BL/6-*Irfng*<sup>tm1</sup> (C57BL/6.IFN- $\gamma$  receptor KO, H-2<sup>b</sup>) mice were all purchased from The Jackson Laboratory. F1 generation mice between the C57BL/6 and B10.D2 (B6D2F1), the C57BL/6-*Irfng*<sup>tm1Ts</sup> and BALB/c-*Irfng*<sup>tm1Ts</sup> (CB6F1.IFN- $\gamma$ KO), and the BALB/c.RAG1<sup>-/-</sup> and C57BL/6.RAG1<sup>-/-</sup> (CB6F1.RAG1<sup>-/-</sup>) strains were bred in our laboratory. C57BL/6.CD1d<sup>-/-</sup> and CB6F1.CD1d<sup>-/-</sup> mice were a gift from Dr. Mark Exley (Harvard Medical School, Boston, MA). All mice were kept in filter-top cages and remained entirely healthy throughout the experiments. All animals were cared for according to American Association for the Accreditation of Laboratory Animal Care-approved methods.

### Murine heart transplantation techniques

Mouse hearts were transplanted to a heterotopic abdominal location with appropriate microsurgical anastomoses according to our previously described technique (15). The continuing status of transplanted hearts was determined by direct palpation at least twice per week with the vigor of contractions of the transplants being recorded on a scale of 0–3+.

### Antibodies

mAbs against mouse CD4 (GK1.5, a rat IgG2b) and CD8 (2.43, a rat IgG2b) were produced from cell clones acquired from American Type Culture Collection. Anti-mouse NK1.1 mAb (PK136, a mouse IgG2a) was purchased from Bio Express. Abs used in immunohistochemistry included anti-mouse CD11b mAb (M1/70; Boehringer-Mannheim), anti-CD3 (YCD3–1; C. A. Janeway, Yale University, New Haven, CT), and anti-Ly49G2 (4D11; American Type Culture Collection). To deplete target cells *in vivo*, mAbs were delivered by intraperitoneal injections of 0.2 ml of combined ascites fluid containing anti-CD4 and anti-CD8 mAbs, or 200  $\mu$ g anti-NK1.1 mAb, or both on days –7, –1, and +1 relative to the day of transplantation, followed by weekly injections until postoperative day 56.

### Histological techniques

Transplanted hearts were typically removed from recipients on postoperative day 56, and frozen sections were prepared to determine the presence and degree of CAV formation as described previously (5). Intimal proliferative changes were classified into three stages, as reported previously in detail (5). Immunopathological analysis was performed as in previous studies (16). In brief, 4- $\mu$ m cryostat sections were stained with mAbs directed to mouse CD3, CD4, CD8, ASGM1, Mac1, and Ly49G2. The distribution and intensity of the infiltrate was noted for each of the markers.

### Preparation of CD4<sup>+</sup> T cells and adoptive transfer experiments

CD4<sup>+</sup> T cells were collected from CB6F1 splenocytes by magnetic cell sorting using the CD4<sup>+</sup> T cell isolation kit (Miltenyi Biotec) according to the manufacturer's protocols, with a resulting purity of >85% as assessed by flow cytometry. A total of  $5 \times 10^6$  CD4<sup>+</sup> T cells were adoptively transferred into CB6F1.RAG1<sup>–/–</sup> recipients on the day of transplantation. CD4<sup>+</sup> T cells were still surviving in peripheral blood and spleen on the day the mice were killed (data not shown).

### YAC-1 targeted <sup>51</sup>Cr release assay

NK cytotoxicity in splenocytes was measured with a standard 4-h <sup>51</sup>Cr release assay using YAC-1 target cells as described previously (17). Thirty-five days after transplant, splenocytes were collected from CB6F1 recipients of either CB6F1 or C57BL/6 hearts. These splenocytes were cocultured for 4 h with <sup>51</sup>Cr-labeled YAC-1 cells in triplicate to yield desired E:T ratios (e.g., 50:1, 25:1, 12.5:1, 6.25:1). Collected supernatants were counted using a gamma counter. Culture medium alone or HCl (1 mol/L) was added to labeled target cells for calculation of spontaneous release or maximum release, respectively. The percentage of lysis was calculated using the following equation: % lysis = [(Experimental release – spontaneous release)/(maximum release – spontaneous release)]  $\times$  100.

### MLR assay

Splenocytes were cultured in triplicate in 96-well flat-bottom plates containing  $4 \times 10^5$  recipient splenocytes as responders with  $4 \times 10^5$  donor splenocytes as stimulators. Stimulator cells received 3 Gy from a <sup>137</sup>Cs

source while suspended in 10% FCS-RPMI 1640 medium at 37°C in CO<sub>2</sub> for 3 days before they were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine/well, followed by harvesting ~6 h later. Stimulation indices were calculated by dividing mean cpm in anti-donor, anti-self, and anti-third-party responses by the result of mean cpm minus background cpm (i.e., cpm with no stimulator cell population) (18).

### Flow cytometric analysis

Recipient splenocytes were prepared as described above. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2, and the splenocytes were then washed and incubated with PE-conjugated anti-pan-NK (DX5) and Cy-conjugated anti-CD3e (2C11). All reagents used for blocking and staining were obtained from BD Pharmingen. All incubations were performed for 20 min at 4°C. Lymphocytes were analyzed on a FACScan (BD Biosciences).

### Statistical analysis

Significant differences between group means were determined using Fisher's exact test. A *p* value of <0.05 was considered significant.

## Results

### Parental to F1 transplants develop CAV but not acute rejection

It has been widely accepted that organs transplanted from parental donors to F1 hybrid recipients, as distinguished from bone marrow transplants, survive indefinitely without evidence of acute rejection due to the absence of a host adaptive immune response. Accordingly, when we transplanted C57BL/6 mouse hearts into (C57BL/6  $\times$  BALB/c)F1 recipients (hereafter CB6F1) that received no immunosuppression, all transplanted hearts continued to beat vigorously throughout the 56-day observation period in a manner entirely similar to isografts (Table I). Fully allogeneic C3H/HeJ hearts were rejected within 15 days, as expected (Table I, group 7).

Although parental to F1 heart transplants were indistinguishable on gross inspection from isografts when removed at 56 days, histological examination revealed impressive and advanced CAV in the coronary vessels of 19 of 22 transplanted parental hearts (Table I, group 1). These arterial lesions exhibited both collagen and smooth muscle cell accumulation (Fig. 1*a*) that, in some cases, resulted in nearly complete luminal occlusion (Fig. 1*c*). Cuffs of infiltrating cells were present around affected vessels, although there was little or no accompanying inflammation in the myocardium. These lesions reproduced with fidelity the vascular lesions observed in human heart transplant recipients undergoing chronic rejection. Similar lesions were observed in two additional parental to F1 strain combinations, C57BL/6 into (C57BL/6  $\times$  B10.D2)F<sub>1</sub> and C57BL/6 into (C57BL/6  $\times$  C3H/HeJ)F<sub>1</sub> (Table I, groups 2 and 3, respectively). The observed strain difference in the frequency and severity of lesion formation correlates well with published strain-specific variations in the strength of hybrid resistance

Table I. Allograft vasculopathy in parental to F1 heart transplants

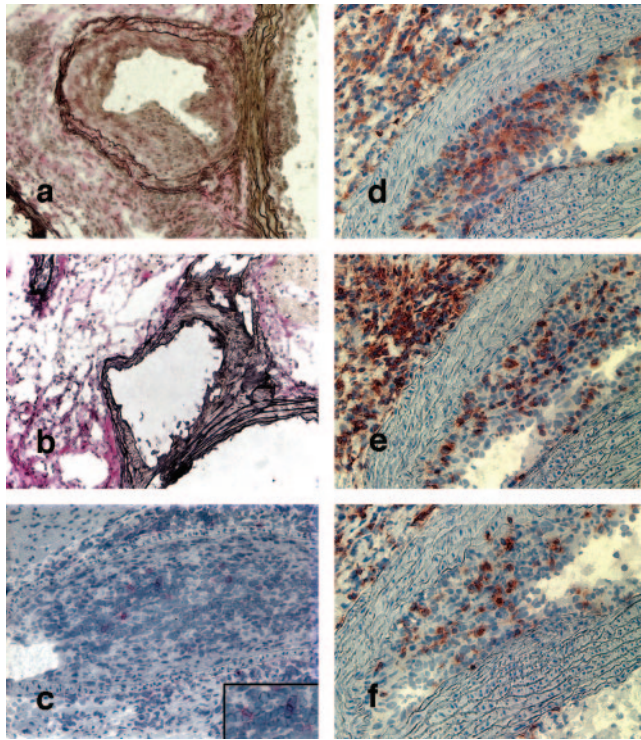
Group	Donor	Recipient	Palpation Score <sup>a</sup>	Stage of CAV <sup>b</sup>	Hearts with CAV
1	C57BL/6	(C57BL/6 $\times$ BALB/c)F <sub>1</sub>	2.8	2.3	19/22 <sup>c</sup>
2	C57BL/6	(C57BL/6 $\times$ B10.D2)F <sub>1</sub>	3.0	1.5	3/4 <sup>c</sup>
3	C57BL/6	(C57BL/6 $\times$ C3H/HeJ)F <sub>1</sub>	2.3	0.4	3/9
4	C57BL/6	C57BL/6	2.8	0	0/7
5	(C57BL/6 $\times$ BALB/c)F <sub>1</sub>	(C57BL/6 $\times$ BALB/c)F <sub>1</sub>	2.8	0	0/9
6	(C57BL/6 $\times$ B10.D2)F <sub>1</sub>	(C57BL/6 $\times$ B10.D2)F <sub>1</sub>	3.0	0	0/3
7	C3H/HeJ	(C57BL/6 $\times$ BALB/c)F <sub>1</sub>	N/A	N/A	Acute rejection

N/A, Not applicable.

<sup>a</sup> Average group palpation score, graded from 0 to 3, determined at the time the mice were killed (day 56).

<sup>b</sup> Average group severity score, graded from 0 to 3, determined at the time the mice were killed (day 56).

<sup>c</sup> *p* < 0.05 by Fisher's exact test compared with group 4.



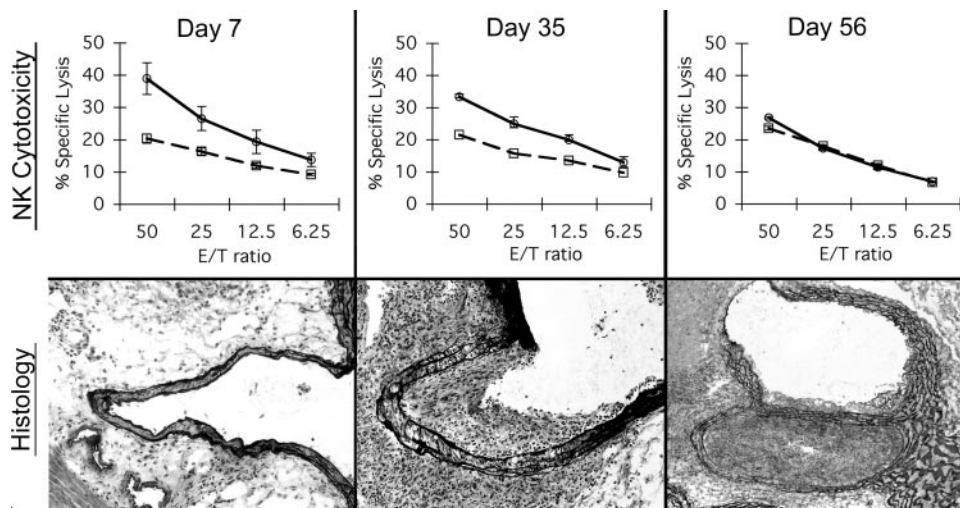
**FIGURE 1.** Immunohistochemistry of proximal coronary arteries from parental hearts transplanted into F1 recipients. *a*, C57BL/6 hearts transplanted into CB6F1 recipients developed florid CAV by day 56. This lesion is representative of all CAV encountered in our experimental groups. *b*, CB6F1 isografts did not develop vascular lesions. *c*, A lesion from a C57BL/6 heart transplanted into a CB6F1 recipient 56 days earlier stained with Ly49G2 mAb reveals a scattered NK cell infiltrate (see inset). Lesions from the same strain combination (day 35 after transplant) stained with mAbs to Mac1 (*d*), CD3 (*e*), and CD8 (*f*) ( $\times 200$ ).

(19). In contrast, no CAV appeared in 19 concurrent heart isografts performed within the three strain combinations (Table I, groups 4–6, and Fig. 1*b*).

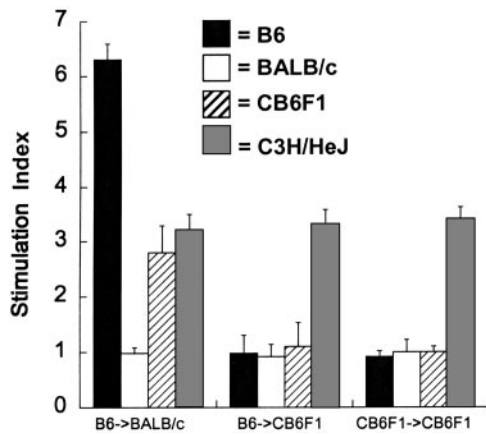
Immunohistological evaluation of the CAV lesions revealed a rich cellular infiltrate in the intima and adventia that included scattered Ly49G2-positive NK cells (Fig. 1*c*). The predominant cells were macrophages (Fig. 1*d*) and CD3<sup>+</sup> T cells (Fig. 1*e*), primarily of the CD8 phenotype (Fig. 1*f*), which localized to the intima and adventia of the donor artery. Vascular lesions were observed in the distal intramyocardial arteries to a lesser degree, similar to the distribution noted previously (8). To estimate the timing of lesion formation, F1 hybrid recipients were killed at 7, 21, and 35 days after transplant. In the C57BL/6 to CB6F1 combination, cellular lesions became evident at 21 days but were not detected at 7 days (Fig. 2).

*Parental to F1 transplants stimulate NK responses but not specific anti-donor T cell activity*

To document NK cell activation in parental to F1 solid organ transplantation, we measured the cytotoxicity of recipient NK cells against YAC-1 targets. Splenic NK cells from CB6F1 recipients receiving C57BL/6 hearts 7 days earlier demonstrated significantly greater cytotoxicity compared with NK cells from CB6F1 isograft recipients (Fig. 2), consistent with the predictions made by the missing self hypothesis. NK cell activation remained elevated in the F1 hybrid recipients of parental grafts at day 35 after transplant (Fig. 2). Parenthetically, this increase in cytotoxicity was not due to a change in splenic NK cell numbers because the relative number of DX5<sup>+</sup> CD3<sup>-</sup> NK cells as a percentage of total splenocytes was constant. However, by 56 days, when parental hearts had developed advanced CAV, the level of NK cytotoxicity in parental to F1 hybrid recipients had returned to levels similar to those found in isograft recipients (Fig. 2). This suggests that early NK cell activation in F1 hybrid recipients triggered a pathway that led to mature vascular lesions and that these lesions persisted after the NK cell response was no longer detectable. To document the absence of a specific host anti-donor T cell response in F1 hybrid recipients of parental hearts, T cells from CB6F1 recipients were shown not to respond to donor parental C57BL/6 cells in a one-way MLR (Fig. 3). These results confirm that the parental-to-F1



**FIGURE 2.** NK cytotoxicity and coronary artery histology in parental to F1 strain combinations. YAC-1-targeted NK cytotoxicity assay using splenocytes from CB6F1 recipients bearing C57BL/6 hearts (solid line) or CB6F1 isografts (dashed line) at 7, 35, and 56 days after transplant. Values for each animal were determined as the mean of triplicate wells. Each error bar represents the mean of three animals. Simultaneous histology of coronary arteries emerging from the aorta at these time points shows development of CAV lesions starting after the initial onset of up-regulated NK cytotoxicity. A rich cellular endovascular lesion is evident by day 35 that matures to a more fibrotic stage after NK activity has subsided (day 56). All sections were stained with Weigert's elastin stain. Magnification: day 7,  $\times 200$ ; day 35,  $\times 100$ ; day 56,  $\times 100$ .



**FIGURE 3.** In vitro analysis of T cell reactivity in parental to F1 strain combinations. One-way MLRs using splenocyte responders from recipients used in three different groups of transplants: C57BL/6 (B6) to BALB/c and B6 to CB6F1 and CB6F1 isografts. Stimulators consisted of irradiated B6 splenocytes (filled bars), BALB/c splenocytes (open bars), CB6F1 splenocytes (hashed bars), or C3H/HeJ splenocytes (stippled bars). Each point represents the mean of three animals. Error bars represent 1 SD of results from three different animals.

transplant system induces a vigorous NK cell response but not an obvious specific anti-donor T cell response.

#### *NK-triggered CAV requires the participation of T cells nonspecific for donor MHC but not NKT cells*

Because the classical hybrid resistance phenomenon is mediated only by NK cells (14), the development of florid CAV lesions in F1 hybrid recipients suggested that activated host NK cells, not T or B cells, triggered the pathway leading to the observed intimal proliferation. To test this hypothesis, we performed transplants using RAG1<sup>-/-</sup> donors and/or recipients that are completely deficient in T and B cells while retaining competent NK cells. Whereas transplantation of C57BL/6.RAG1<sup>-/-</sup> hearts into wild-type CB6F1 recipients resulted in fulminant CAV (Table II, group 1), these same C57BL/6.RAG1<sup>-/-</sup> hearts surprisingly failed to develop CAV when transplanted into CB6F1.RAG1<sup>-/-</sup> recipients (Table II, group 2). This finding suggests that NK cells are not fully sufficient to provoke CAV formation in parental to F1 transplants. However, CAV lesions were reconstituted when wild-type naive CB6F1 CD4<sup>+</sup> T cells were adoptively transferred into CB6F1.RAG1<sup>-/-</sup> recipients of C57BL/6.RAG1<sup>-/-</sup> hearts (Table II, group 3), suggesting that CD4<sup>+</sup> T cells contribute to the development of CAV in this system rather than B cells. This conclusion was further supported by the absence of C4d immunoperoxidase staining in parental to F1 allografts, because this complement component is typically deposited in tissue when Ab-mediated rejection occurs (data not shown).

These findings do not completely exclude the possibility that T cells are capable of fueling CAV development in the parental to F1 transplant system entirely independent of NK cell involvement.

However, the adoptively transferred CB6F1 CD4<sup>+</sup> T cells lacked specificity against parental donor MHC molecules as confirmed by MLR assays, and they were therefore likely to require recruitment and activation by a trigger cell population such as NK cells. To confirm the involvement of NK cells in this CAV cascade, as well as to explore the potential interaction between NK and T cells, we used depleting Abs in the original C57BL/6 to CB6F1 hybrid strain combination. In contrast to the parental to F1 hybrid transplant experiments in RAG1<sup>-/-</sup> mice (Table II, group 2), depletion of T cells alone in wild-type recipients with anti-CD4 and anti-CD8 mAbs failed to prevent CAV lesion development (Table III, group 2). The discordant finding of CAV formation in T cell-depleted wild-type recipients compared with RAG1<sup>-/-</sup> recipients may be attributed to a threshold effect, because ~5–10% of CD3<sup>+</sup> T cells remained after depletion with mAbs (data not shown).

Although T cell depletion alone could not prevent CAV, combined T cell and NK cell depletion with anti-CD4, anti-CD8, and anti-NK1.1 mAbs resulted in a significant abrogation in lesion development (Table III, group 3). Furthermore, using T cell subset-specific mAbs, we confirmed that the CD4<sup>+</sup> T cell population and not the CD8<sup>+</sup> T cell subset was required for the generation of CAV in this system (Table III, groups 4 and 5). The efficacy of adding NK cell depletion to these T cell-depleting regimens is especially remarkable considering the relatively incomplete depletion of NK cells achieved by anti-NK1.1 mAb in this transplant system, with up to 20% of DX5<sup>+</sup>CD3<sup>-</sup> NK cells persisting after anti-NK1.1 treatment (data not shown). This lack of complete NK cell depletion may explain the ineffectiveness of treatment with anti-NK1.1 mAb alone, which did not prevent CAV in parental to F1 transplants (Table III, group 1). To confirm and extend these findings, we repeated the depletion experiments in a strain combination mismatched for the full MHC. Again, the combination of NK cell depletion with T cell depletion led to a significant reduction in CAV compared with treatment with T cell depletion alone (Table III, groups 6–9).

Because the NK1.1 marker is also expressed by invariant NKT cells, we sought evidence for their role in the pathogenesis of CAV using parental to F1 cardiac transplants in CD1d<sup>-/-</sup> mice that are completely NKT cell deficient. These transplanted hearts all developed CAV despite the absence of NKT cells, indicating that NK cells rather than NKT cells contribute to CAV formation (Table III, group 10).

Together, these Ab depletion studies suggest that there is a necessary interaction between NK cells and T cells in the formation of CAV in this system, consistent with the observed prominent infiltrate of both NK and T cells in the lesions. This interaction may be mediated by either soluble signals such as cytokines or by direct cell-cell receptor engagement.

#### *IFN-γ is required for CAV development in a parental to F1 hybrid transplant system*

The known association between IFN-γ and CAV (16, 20) and the recognized capacity of NK cells to produce IFN-γ (16, 21) suggested that this cytokine could play an important role in the NK

Table II. Role of T cells in the development of CAV in parental to F1 transplants

Group	Donor	Recipient	Treatment	Hearts with CAV
1	C57BL/6.RAG1 <sup>-/-</sup>	CB6F1	None	5/5 <sup>a</sup>
2	C57BL/6.RAG1 <sup>-/-</sup>	CB6F1.RAG1 <sup>-/-</sup>	None	0/9
3	C57BL/6.RAG1 <sup>-/-</sup>	CB6F1.RAG1 <sup>-/-</sup>	CD4 <sup>+</sup> T cells <sup>b</sup>	3/5 <sup>a</sup>

<sup>a</sup>  $p < 0.05$  by Fisher's exact test compared with group 2.

<sup>b</sup> Adoptive transfer of  $5 \times 10^6$  wild-type CB6F1 CD4<sup>+</sup> T cells on the day of transplant.

Table III. *Effects of NK1.1<sup>+</sup> cell and T cell depletion*

Group	Strain Combination <sup>a</sup>	Depletion <sup>b</sup>	Hearts with CAV
1		NK1.1 <sup>+</sup> cell	5/5
2		CD4 <sup>+</sup> /CD8 <sup>+</sup> T cell	5/5
3	Parental to F1	NK1.1 <sup>+</sup> cell CD4 <sup>+</sup> /CD8 <sup>+</sup> T cell	2/9 <sup>c</sup>
4		CD4 <sup>+</sup> T and NK1.1 <sup>+</sup> cells	0/5 <sup>c</sup>
5		CD8 <sup>+</sup> T and NK1.1 <sup>+</sup> cells	4/5
6		None	NA <sup>d</sup>
7	Full MHC mismatch	NK1.1 <sup>+</sup> cell	NA <sup>d</sup>
8		CD4 <sup>+</sup> /CD8 <sup>+</sup> T cell	9/11
9		NK1.1 <sup>+</sup> cell CD4 <sup>+</sup> /CD8 <sup>+</sup> T cell	3/10 <sup>e</sup>
10	NKT-deficient parental to F1	None	4/4

<sup>a</sup> Strain combinations: parental to F1 represents C57BL/6 to CB6F1. Full MHC mismatch represents C3H/HeJ to CB6F1. NKT-deficient parental to F1 represents B6.CD1d<sup>-/-</sup> to CB6F1.CD1d<sup>-/-</sup>.

<sup>b</sup> NK cell depletion could not be fully achieved, because ≈20% of NK cells persisted despite NK1.1 depletion. T cell depletion was >90%.

<sup>c</sup>  $p < 0.01$  by Fisher's exact test compared with Table 1, group 1.

<sup>d</sup> Hearts rejected acutely with mean graft survival <9 days. Eight transplants per group. NA, Not applicable.

<sup>e</sup>  $p < 0.05$  by Fisher's exact test compared with Table 3, group 8.

cell-triggered pathway toward CAV. To test this hypothesis, we bred C57BL/6 and BALB/c strains of IFN- $\gamma$ KO mice together to generate F1 hybrids deficient in IFN- $\gamma$  (designated CB6F1.IFN- $\gamma$ KO). When wild-type C57BL/6 hearts were transplanted into CB6F1.IFN- $\gamma$ KO recipients, 0 of 11 hearts developed CAV (Table IV, group 1). To determine the source of IFN- $\gamma$  necessary for lesion formation, we eliminated the ability of donor-derived cells to produce IFN- $\gamma$  by transplanting hearts from C57BL/6 IFN- $\gamma$  knockout mice (C57BL/6.GKO) into unmodified CB6F1 recipients. This failed to reduce the severity of CAV in five of five hearts as all developed florid CAV lesions (Table IV, group 2). Then, to determine the target of IFN- $\gamma$  action, IFN- $\gamma$  receptor knockout mice were used as donors to wild-type F1 hybrid recipients. This also failed to reduce the severity of CAV as four of four hearts clearly developed vascular lesions (Table IV, group 3). Together, these results suggest that recipient-derived IFN- $\gamma$  plays a critical role in the pathogenesis of CAV in parental hearts transplanted into F1 recipients, but that it does so indirectly, perhaps by activating recipient effector cells rather than acting directly on the donor allograft endothelium.

## Discussion

In this study, we demonstrate that advanced CAV lesions develop in cardiac allografts transplanted from parental to unmanipulated F1 hybrid mice, a transplant system that lacks specific anti-donor T cell reactivity but retains anti-donor NK cell responses. This finding, coupled with our ability to prevent CAV lesion formation by a treatment regimen that includes anti-NK1.1 mAb, argues that NK cells activated by the absence of self MHC class I molecules on donor endothelium participate in the pathogenesis of CAV. Although it seems that NK cells alone are not sufficient to induce CAV in this system, they appear to be capable of inciting a cascade of events mediated by T cells nonspecific for donor MHC, which culminates in endothelial injury and CAV formation. A possible role for Abs creating the vascular lesions under study here, although perhaps unlikely, is now being explored. An early search

for the deposition of complement components (specifically C4d) has so far been negative.

These results add a third pathway to the known T and B cell-triggered pathways of CAV. In doing so, they may explain the puzzling results of our previous studies that showed that CAV, a MHC-driven alloimmune process, developed in specifically tolerant recipients (8, 22).

Although no previous reports have attributed a role in CAV pathogenesis to NK cells, several recent studies suggest that NK cells can contribute to the alloresponse against solid organs (23, 24). Perhaps most notably, Maier et al. (24) showed that NK cells appear to provide an alternative source of T cell help and thus contribute to the acute rejection of wild-type hearts transplanted into CD28-deficient mice. Our results extend these findings, demonstrating that NK cells contribute to chronic alloresponses such as CAV formation as well as the acute rejection shown by Maier et al. (24). Importantly, NK cells do not seem sufficient by themselves to mediate either acute or chronic alloresponses, instead requiring functional interactions with T cells in both situations to promote these processes.

Our finding that CAV develops in parental to F1 hybrid transplants also marks the first demonstration of hybrid resistance in solid organ allografts. Historically, the hybrid resistance phenomenon was only noted in bone marrow transplants and transplantable lymphomas. Previous attempts to demonstrate hybrid resistance in parental to F1 transplants of solid organs focused solely on acute rejection alloresponses, the absence of which led to the impression that hybrid resistance did not occur in solid organ allografts (25). Similar to hybrid resistance in bone marrow or tumor transplants, the development of CAV in parental to F1 cardiac transplants requires NK cell activity. However, whereas NK cells are sufficient to cause classical hybrid resistance (14), our results suggest that hybrid resistance in cardiac allografts also requires the recruitment of T cells nonspecific for donor MHC. A possible explanation for this discrepancy is that NK cells possess sufficient effector mechanisms to mediate the cytolysis and rejection of both bone marrow allografts and tumor cells, whereas the more complex pathophysiology of CAV necessitates the recruitment of other intermediary and effector cell populations (such as T cells) to produce the growth factors that drive neointimal cell proliferation.

The requirement of these functional interactions between NK cells and T cells to promote CAV formation was unexpected, given that recipient T cells in the parental to F1 hybrid transplant system are nonreactive toward donor MHC, as confirmed by MLR assays.

Table IV. *Recipient IFN- $\gamma$  production is critical for CAV formation in allografts*

Group	Donor	Recipient	Hearts with CAV
1	C57BL/6	CB6F1.IFN- $\gamma$ KO	0/11
2	C57BL/6.IFN- $\gamma$ KO	CB6F1	5/5
3	C57BL/6.IFN- $\gamma$ R.KO	CB6F1	4/4

However, several hypotheses can be offered to explain how activated NK cells can recruit these seemingly non-alloreactive T cells. First, damage initiated by NK cell attack of the allograft may unveil latent epitopes, such as cardiac myosin, that can be recognized by autoreactive T cells in the recipient and lead to chronic rejection (26). Second, our results might suggest the activation of bystander T cells by NK cells independent of TCR ligation, as has recently been described (27). Finally, it has been demonstrated that inflammatory mediators produced by NK cells can activate recipient NKT cells, a regulatory T cell population not restricted to conventional MHC class I products, which then may serve as downstream mediators due to their cross-talk with NK cells and their ability to recruit other cellular effectors (28, 29). However, our demonstration that CAV still develops in parental to F1 transplants devoid of NKT cells discounts this last hypothesis. Current experiments in our laboratory are aimed at defining the precise mechanisms of T cell recruitment by anti-donor NK cells.

This immunological cross-talk between NK cells and other participants in the pathogenesis of CAV likely involves either soluble mediators (such as the IFN- $\gamma$  abundantly produced by NK cells) or direct cell-cell interactions such as in the OX40/OX40L system (30). In support of the cytokine hypothesis, parental to F1 hybrid transplants using either IFN- $\gamma$ -deficient recipients or donors revealed that IFN- $\gamma$  production by recipient cells was essential for CAV development, whereas donor-derived IFN- $\gamma$  was not required. Although this critical IFN- $\gamma$  is likely produced by recipient NK cells given their ability to release abundant quantities of this cytokine when activated (16, 21), the production of this IFN- $\gamma$  by other recipient cell populations (e.g., T cells) cannot be fully excluded. Additionally, we found that IFN- $\gamma$  could not act directly on the donor endothelium to promote CAV lesion formation, because allografts from donors lacking the IFN- $\gamma$  receptor still developed ubiquitous CAV. Thus, in contrast to other systems (31), recipient-derived IFN- $\gamma$  in our model seems to contribute to CAV pathogenesis indirectly by stimulating downstream effectors and intermediary cell populations rather than by directly promoting donor endothelial cell damage and CAV formation. IFN- $\gamma$  may therefore serve as the missing link between NK cells and non-allogeneic T cells, because Kamath et al. (27) specifically demonstrated that NK-derived IFN- $\gamma$  can trigger T cell activation independent of TCR ligation.

Together, these findings suggest a novel pathway toward CAV development that is distinct from previously characterized pathways dependent exclusively on T or B cells. This novel pathway is instead triggered by recipient NK cells activated by the absence of recipient MHC molecules on the allograft endothelium. Although allograft myocardial cells also lack recipient MHC products, the activity of recipient NK cells seems focused predominantly on the allograft endothelium, consistent with previous studies suggesting that activated endothelial cells are particularly susceptible to NK cell attack due to endothelial expression of the CX3C chemokine fractalkine (32). Likely through their release of IFN- $\gamma$ , activated NK cells recruit other downstream intermediary and effector cell populations in the recipient, including CD4<sup>+</sup> T cells nonspecific for donor MHC. This cascade of immunological cross-talk finally culminates in donor endothelial cell damage and CAV formation.

Although this pathway is perhaps best supported by the available data, at least two alternate hypotheses may also be raised to explain the occurrence of CAV in parental to F1 transplant systems. The first alternate hypothesis proposes that CAV in this system is due to a chronic graft-vs-host response triggered by passenger leukocytes carried by the donor cardiac allograft. Whereas recipient T cells lack anti-donor specificity, donor T cells should have anti-recipient reactivity, and if enough of them were carried

into the recipient with the allograft, they could theoretically trigger a graft-vs-host response, establishing a chronic low level of inflammation that could promote CAV formation, completely independent of recipient NK activity. However, this hypothesis is substantially refuted by the development of CAV in wild-type CB6F1 recipients of C57BL/6.RAG1<sup>-/-</sup> hearts, which completely lack passenger leukocytes (Table II, group 1). A second alternate hypothesis contends that two parallel redundant pathways exist that both independently lead to CAV formation in parental to F1 transplant systems, one T cell mediated and the other dependent on NK cells. This hypothesis is bolstered by the Ab depletion data, which found that partial depletion of either NK or T cells alone failed to prevent CAV, whereas combined depletion of NK and T cells did eliminate CAV. However, this hypothesis is negated by the failure of CAV to develop in C57BL/6.RAG1<sup>-/-</sup> to CB6F1.RAG1<sup>-/-</sup> transplants (Table II, group 2), because this combination should possess an intact NK pathway even though the T cell pathway is inactive.

These experiments add new insights into the ill-defined pathogenesis of chronic rejection by implicating NK cells in the formation of CAV, a finding likely to have direct clinical relevance and significance. Supporting the clinical implications of this study, NK cells do infiltrate cardiac allografts and are known to accumulate in CAV lesions in humans (33). Also, NK cell-mediated cytolysis of donor target cells in vitro is increased in NK cell populations isolated from recipients of allogeneic heart transplants (34). A possible role for NK cells in nontransplant atherosclerosis (35, 36) might be suspected from the known association between viral infections and atherosclerosis (37) and the recognized ability of viruses to down-regulate MHC class I molecules (38). Because NK cells are not targeted by current immunosuppressive therapy, including cyclosporine (24), the current results may explain why CAV, a primarily MHC-driven alloimmune process, still occurs in heavily immunosuppressed recipients. Indeed, the apparent resistance of NK cells to the modulating effects of conventional immunosuppression is compatible with the hypothesis that innate immunity may be an important part of the complex set of events that result in this vexing problem. These results suggest that NK cell inactivation or depletion in humans may improve the long-term outcome of transplanted organs.

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## Disclosures

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