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Depletion of T Regulatory Cells Promotes Natural Killer Cell-Mediated Cardiac Allograft Vasculopathy

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Abstract

Background—A role for NK cells in cardiac allograft vasculopathy (CAV) was suggested by our earlier observation that CAV arises even in the absence of detectable anti-donor T or B cell reactivity in parental to F1 mouse heart grafts. However, prevention of CAV in this setting required the depletion of both NK and CD4⁺ T cells.

Methods—To clarify the interrelationship between NK and CD4⁺ cells we analyzed early events and selective depletion of T regulatory cells (Tregs). Hearts from C57BL/6 (B6) donors were transplanted heterotopically into BALB/c × C57BL/6 (CB6F1) recipients and NK cells, CD4⁺ T cells, and/or Tregs were depleted with anti-NK1.1 (PK136), anti-CD4 (GK1.5) or anti-CD25 (PC61), respectively.

Results—In contrast to prior studies in which the prevention of CAV at 8 weeks required the co-depletion of NK and CD4⁺ T cells, NK cells depletion alone eliminated CAV at 3 weeks.

Furthermore, depletion of CD25⁺ cells accelerated the onset and maturation of CAV at both 2 and 3 weeks ($p < 0.02$ and $p < 0.001$, respectively). However, anti-NK1.1 treatment prevented lesions in CD25-depleted recipients. Finally, CD4⁺ T cell depletion alone did not prevent or accelerate development of CAV but inhibited the effect of CD25⁺ T cell depletion.

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Conclusion—These data suggest that NK cells can play an important role in the early pathogenesis of CAV but that their ability to mediate early CAV can be modulated by Tregs.

Keywords

Cardiac allograft vasculopathy; heart transplantation; NK cells; Tregs; mice

Introduction

The utility of cardiac transplantation is limited by the development of CAV, the leading cause of mortality after the first post-transplant year [1]. CAV is characterized by cellular infiltration and proliferation of cells forming a neointima that culminates in luminal occlusion, ischemic injury, fibrosis of myocardium, and eventual allograft failure [2, 3]. Despite extensive investigation, the underlying mechanisms responsible for CAV remain poorly defined. Experimental studies, especially in mice have shown that CAV may be mediated by T cells [4], alloantibody [5, 6], macrophages [7], NK cells [8–10].

Using a parental to F1 heart transplant system which eliminates ordinary host anti-donor T and B cells responses while maintaining normal host NK responses, we have demonstrated that NK cells contribute to the development of CAV [8]. Curiously, prevention of CAV at 8 weeks post-transplantation was not achieved by depletion of NK cells alone, but required the concurrent depletion of CD4⁺ T cells but not CD8⁺ T cells [8]. This result suggested the involvement of an alternative CD4⁺ T cell-dependent process that was apparently directed against self-antigens.

To understand how NK cells and T cells relate in the pathogenesis of CAV, we selectively depleted NK cells and/or T cells early after transplantation when NK cell activity was greatest [8]. Our results suggest that in contrast to the later phases of CAV, the early phase of CAV in the parental to F1 system is primarily NK cell dependent, but that CD25⁺ Tregs are able to modulate the development of early NK cell-mediated CAV through indirect effects on effector CD4⁺ T cells. These findings demonstrate the close interaction between the innate and adaptive immune systems in regulating the chronic rejection response to an allograft.

Results

NK cells depletion alone prevents the early lesions of CAV

We have previously shown that in a parental to F1 transplant system, depletion of both NK cells and CD4⁺ T cells is required to prevent the development of CAV at late time points defined as 8 weeks or more posttransplantation [8]. However, *in vitro* studies demonstrated that NK cell activity in spleens of F1 recipients of parental hearts peaked 4 weeks posttransplantation and returned to baseline by 8 weeks [8]. We postulated that NK cells have their greatest effect on the development of CAV within the first 4 weeks post transplant.

To test this hypothesis, parental B6 hearts were transplanted into CB6F1 recipients and explanted for evaluation at 3 weeks instead of 8 weeks. In the untreated control group, 6 of 8

hearts developed CAV (Table 1, Group 1). However, when recipients were treated with anti-NK1.1 mAb to deplete NK cells, CAV was prevented in every allograft (Table 1, Group 2). These findings suggest that, in contrast to later phases, the early phase of CAV in this system is primarily dependent on NK cell activity.

Depletion of CD4⁺CD25⁺ T cells increases CAV lesion size and cellularity

Although T cells do not appear to play a direct role in the early pathogenesis of CAV, they could affect lesion formation indirectly by either enhancing or inhibiting NK cell activity. Indeed, it is known that CD4⁺CD25⁺ Treg can suppress the *in vivo* activity of NK cells through a TGFβ-dependent pathway [11–13]. To test the hypothesis that host Tregs play an inhibitory role in early, NK-mediated CAV, we treated CB6F1 recipients of B6 hearts with PC61 to deplete CD4⁺CD25⁺ Tregs.

Allografts explanted from CD25⁺ T cell-depleted recipients at 3 weeks exhibited a similar frequency of CAV to allografts explanted from untreated controls (12/12 versus 6/8) (Table 1, Group 3). However, when examined histologically, the coronary lesions in the CD25⁺ T cell-depleted recipients demonstrated much greater cellularity than the lesions observed in the untreated recipients suggesting that these lesions were more advanced or mature than the lesions observed in untreated recipients (compare Fig. 1A and 1B). Furthermore, the median neointimal index (% stenosis) of the proximal coronary arteries in heart allografts explanted from anti-CD25 treated recipients was significantly greater (82.1%) than that observed in hearts from untreated B6 to CB6F1 controls (23.4%) (Fig. 2A). Of note, CB6F1 recipients of CB6F1 isografts treated with anti-CD25 mAb remained free of disease (Table 1, Group 4).

Depletion of CD25⁺ T cells accelerates the development of CAV

The advanced stage and size of vascular lesions seen in anti-CD25 mAb-treated recipients suggested that CD25⁺ T cell depletion accelerated the development of CAV. To test this hypothesis, heart allografts were explanted from anti-CD25 mAb-treated recipients at 2 weeks instead of 3 weeks. B6 hearts transplanted into untreated CB6F1 recipients and removed at 2 weeks showed no lesions (Table 1, Group 5). In contrast, allografts from 6 of 7 recipients treated with anti-CD25 mAb showed accelerated CAV at 2 weeks with a median intimal index of 42.3% (Table 1, Group 6).

NK cells and CD4⁺ T cells infiltrate the neointima of hearts from Treg depleted recipients

Occasional NK cells were detected in the early CAV lesions present in allografts from untreated controls at three weeks (Fig. 2B, Control). In contrast, there was a much greater NK cell infiltrate in the neointima of hearts from Treg depleted recipients (Fig 2B, α-CD25). Other abundant cell types detected in CAV lesions in Treg depleted recipients were CD3⁺CD4⁺ T cells (Fig. 3a and 3b). CD8⁺ cells were not identified. CD11c⁺ cells, probably dendritic cells, were also prominent, as were CD16⁺ cells (NK cells and/or macrophages) (Fig 3c and 3d, respectively). Of note, the interaction of CD4⁺ T effector cells and FoxP3⁺ Tregs may occur away from the vessel wall as we did not observe the localization of Foxp3⁺ T cells in the arterial walls of allografts in either untreated animals (Fig 4) or anti-CD25-treated animals (Fig 4).

NK cell depletion eliminates the effects of anti-CD25 mAb on lesion formation

To determine whether the exacerbation of CAV by anti-CD25 treatment was NK cell-dependent, recipients were treated with a combination of anti-CD25 and the anti-NK1.1 mAbs, and sacrificed at 3 weeks. Table 1 shows that NK cells depletion completely prevented the development of CAV in anti-CD25 treated recipients (Table 1, Group 7; Fig 1C and Fig 2A). Additionally, anti-CD25 and the anti-NK1.1 mAbs resulted in the absence of CD3⁺ and CD4⁺ cells in the lesions (Fig. 4). These data suggest that CD25⁺ Tregs can affect the severity of CAV and is modulated by NK cells, since the depletion of NK cells results in the elimination of anti-CD25 mAb effect on the cardiac allografts.

Anti-CD4 mAb treatment inhibits the augmentation of CAV by Treg depletion

Based on our previous results with CD4⁺CD25⁺ T cell depletion, we expected that depletion of the full CD4⁺ T cell subset would lead to accelerated coronary lesions of the parental hearts, as does anti-CD25 mAb because anti-CD4 mAb depletes whole CD4⁺ T cells including CD4⁺CD25⁺ T cells. However, anti-CD4 mAb therapy neither augmented nor abrogated CAV (Table 1, Group 8).

Furthermore, since anti-CD25 mAb is IgG2a, some cell types, especially NK cells, could react with this depleting mAb through Fc receptors. In order to rule out a possibility due to antibody mediated responses of NK cells through Fc receptor, anti-CD25mAb and anti-CD4mAb were given on days -7 and -1 +5 and weekly thereafter and on days -6, -3, -1, +1 and weekly thereafter, respectively. When anti-CD4 mAb was given concurrently with anti-CD25 mAb, allografts still developed CAV (Table 1, Group 9) similar in frequency to that seen of untreated recipients but the severity of the lesions was significantly less than that in hearts from recipients treated with anti-CD25 mAb alone (Fig. 2A).

Discussion

We have previously demonstrated that advanced CAV lesions develop in cardiac allografts transplanted from parental to unmanipulated F1 hybrid mice, a transplant system of hybrid resistance that lacks specific anti-donor T cell reactivity but retains anti-donor NK cell responses [8]. The appearance of late CAV lesions, defined as those observed 8 weeks following transplantation, was prevented by co-depletion of NK cells and CD4⁺ T cells but not by the depletion of NK cells alone or by the co-depletion of NK cells and CD8⁺ T cells [8]. Thus, while it seemed that NK cells alone were not sufficient to induce CAV in this system, they appeared to be necessary to incite a cascade of events mediated in part by CD4⁺ T cells nonspecific for donor MHC which culminated in endothelial injury and formation of CAV at 8 weeks. This differs from classical hybrid resistance in which NK cells in F1 recipients are capable of rejecting parental bone marrow or tumors with no known participation of T cells.

To further understand the relationship between NK cells and T cells in the pathogenesis of CAV in the *early* stages of the disease, we assessed the effects of NK cells and T cell subset depletion in F1 recipients whose parental hearts were removed and evaluated for CAV 3 weeks post-transplantation, instead of 8 weeks. We chose this earlier time point because our

previous studies showed that NK cell activity, as measured by the cytotoxicity of recipient NK cells against YAC-1 targets, was greatest during the first 4 weeks post transplantation (8).

When parental hearts were transplanted into F1 recipients and removed at 3 weeks, 6 of 8 grafts still displayed lesions of CAV, but the lesions were morphologically immature. When recipients underwent NK cell depletion alone, CAV was prevented in all grafts explanted at 3 weeks. This suggests that the early phase of CAV pathogenesis, which coincides with the period of greatest NK cell activity, is primarily dependent on NK cells. Together, our findings suggest that NK cells are necessary and sufficient to induce the early immature lesions of CAV but that the maturation of vascular lesions into the fulminant form observed at 8 weeks requires the participation of T cells.

Despite the seemingly minor role CD4⁺ T cells played in the early phase of CAV, a recent report showing that depletion of Tregs in F1 mice receiving parental bone marrow transplants promoted NK cell activation and resulted in the accelerated rejection of transplanted marrow cells [11], suggesting that CD4⁺CD25⁺ Tregs could still be playing a role in early NK cell-mediated CAV. To test this hypothesis, we depleted CD25⁺ Tregs in F1 recipients of parental hearts and examined the allografts 3 weeks after transplantation. The CAV lesions were morphologically more advanced than in allografts from untreated controls. Indeed, the acceleration of CAV by Treg depletion was confirmed by the fact that hearts explanted from Treg depleted recipients at 2 weeks exhibited florid CAV lesion whereas allografts from untreated recipients showed no CAV at 2 weeks. The additional finding that NK cell depletion eliminated the effects of Treg depletion on accelerated lesion formation suggested that Tregs were affecting the development of CAV by modulating NK cell activity either directly or indirectly.

To address how Tregs influence NK cell activity in the early phase of CAV, we evaluated the effects of CD4⁺ T cell depletion with and without the co-depletion of CD25⁺ Tregs. Systemic depletion of CD4⁺ T cells following anti-CD4 mAb treatment alone did not affect the frequency or severity of CAV lesions as compared to untreated controls, which was consistent with our finding that NK cells alone predominate in the early pathogenesis of CAV lesion formation. However, when anti-CD4 mAb treatment was combined with anti-CD25 mAb therapy, the depletion of CD4⁺ T cells eliminated the acceleration of lesion formation observed in recipients depleted of Tregs. This suggests that host CD4⁺ T cells participate in the Treg inhibition of NK cell-mediated early CAV even though they do not recognize donor MHC.

One explanation for the participation of donor MHC non-reactive host T cells in early CAV formation may be “bystander” activation, which can occur independent of TCR ligation [14]. An alternative explanation is that autoreactive T cells recognizing latent epitopes such as cardiac myosin, unveiled by procedure-associated ischemia/reperfusion injury, become activated after transplantation [15]. Indeed, Kim and colleagues have shown that depletion of Tregs results in increased autoreactive T cell activity, leading to severe autoimmune responses. Furthermore, the authors observed a 4-fold and 7-fold increase in NK1.1⁺ cells in the spleen and lymph nodes respectively in these Treg depleted animals and a 2-fold increase in TCRβ⁺CD4⁺ cells in both spleen and lymph nodes [16].

Together, our results could be explained as follows. In untreated recipients, NK cells, activated by the absence of host MHC molecules on the allograft endothelium, initiate the pathogenesis of early CAV, perhaps through the production of IFN- γ . Our previous studies [17] and others [18] suggest that recipient-derived IFN- γ is an important mediator of donor endothelial cell damage and early CAV formation. In this situation, host Tregs suppress any potential additional contributions from recipient CD4⁺ T cells, be they autoreactive or bystander in nature. In contrast, when Tregs are depleted, autoreactive or bystander CD4⁺ T cells are recruited to the early pathogenesis of CAV which accelerates the maturation of the vascular lesions. One can envision that the IL-2 produced by activated CD4⁺ T cells upregulates NK cell activity which accelerates lesion maturation [19] or that the additional IFN- γ generated by activated T cells contributes directly to the acceleration of neointimal proliferation. Recently, Gasteiger and colleagues have shown that missing-self responses by NK cells were increased in the absence of Treg cells due to increased IL-2 availability [20]. Consistent with this finding, Sitrin and colleagues found that Treg cells regulate NK cell function by controlling the availability of limiting amounts of IL-2 generated mainly by infiltrating CD4⁺ T cells in the pancreatic insulinitic lesions [21].

In conclusion, our findings suggest that, in contrast to the later phases of CAV, the early phase of CAV in the parental to F1 system is primarily NK cell dependent, but that CD25⁺ Tregs are able to modulate the development of early NK cell-mediated CAV through indirect effects on autoreactive CD4⁺ T cells. To our knowledge, these findings are the first to suggest that Tregs can modulate the activity of NK cells *in vivo* and in doing so, further demonstrate the close interaction between the innate and adaptive immune systems in regulating the chronic rejection response to an allograft. Such detailed information can provide a useful guide for targeted intervention in the development of CAV, especially if the mechanisms by which these complicated interactions between cell populations can be defined.

Materials and Methods

Mice

C57BL/6J (*H-2^b*), BALB/cJ (*H-2^d*) and CB6F1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All mice were maintained under pathogen-free conditions in filter-top cages with an automatic watering system throughout the experiments and were cared for according to methods approved by the American Association for the Accreditation of Laboratory Animals.

Cardiac Transplantation

Mouse hearts were transplanted to a heterotopic abdominal location with appropriate microsurgical anastomoses as previously described [22]. Parental C57BL/6(B6) donor hearts were transplanted into (C57BL/6 $\times$ BALB/c)F1 recipients (designated CB6F1). Transplanted hearts were monitored by direct transabdominal palpation at least twice a week. The vigor of contraction of the transplants was recorded on a scale of 0–3+ [8].

Antibodies and Reagents

mAbs against mouse NK1.1 mAb (PK136, mouse IgG2a), CD25 (PC61, rat IgG1), and CD4 (GK1.5, rat IgG2b) were produced by BioXcell, West Lebanon, NH from cell clones acquired from the American Type Culture Collection. NK cells were depleted with 200 μ g of anti-NK1.1 mAb (PK136) injected IP on days -6, +1, and weekly thereafter. The efficacy of anti-NK1.1 mAb (PK136) was confirmed with FACS analysis and approximately 70 % of NK cells were depleted in peripheral blood. CD25⁺ T cells were depleted with 250 μ g of anti-CD25mAb (PC61) injected IP on days -7 and -1 +5 and weekly thereafter. This treatment resulted in Foxp3⁺ cells decreasing by 44% in peripheral blood and by 49% in lymph nodes as compared to untreated recipients which is consistent with previous reports [23]. CD4⁺ T cells were depleted with 100 μ g of anti-CD4 mAb (GK1.5) injected IP on days -6, -3, -1, +1 and weekly thereafter.

Morphometric analysis

Morphometric analysis was performed on images of coronary arteries near the ostia on tissue sections stained with Weigert's elastic stain as described previously [24]. An image of the most representative section was captured digitally by light microscopy at 100 $\times$, 200 $\times$, or 400 $\times$ magnification. ImageJ (image processing program developed by NIH) was used to demarcate the borders of the lumen and the intima of the artery. One evaluator, who was blinded as to the diagnosis and treatment of heart recipients, demarcated the areas on all the sections. The software then quantitated the manually demarcated luminal and intimal areas. From these area values the 'neointimal index', defined as [intimal area/(luminal + intimal area)] $\times$ 100, was calculated.

Histological techniques

Heart allografts were removed from recipients at days 14 or 21. Frozen sections were stained with Weigert's elastic stain to evaluate the severity of coronary lesions as above. Immunopathological analysis was performed as in previous studies [17]. In brief, 4- μ m cryostat sections were stained with mAbs directed to mouse CD3, CD4, CD8, CD11b, CD11c, Foxp3, and NKp46. Cy 5 was used as a secondary antibody for anti-NKp46 antibody [25].

Flow cytometry

Target cell depletion following anti-CD25 mAb (PC61) or anti-NK1.1mAb (PK136) was assessed in peripheral blood and lymph node compartments. In brief, peripheral blood single suspensions and lymph node single suspensions were prepared. Cells were incubated for 30 min at 4°C. NK cells were stained using PE-conjugated anti-pan-NK (clone DX5) (BD Pharmingen). The cells were washed with FACS medium twice and fixed in 2% paraformaldehyde solution. Lymphocytes were stained in the same manner using FITC-conjugated anti-CD25 (clone 7D4) (BD Bioscience), PE conjugated anti-CD4 (clone RM4-5) (BD Bioscience), Cy-5 conjugated anti-CD4 (cloneGK1.5) (eBioscience) and Cy-conjugated anti-CD3e (clone 2C11) (BD Bioscience). To examine the levels of Foxp3⁺ T cells, PE-conjugated anti-Foxp3 intracellular staining (clone FJK-16s) (eBioscience) was

performed. Lymphocytes were analyzed on a FACScan (BD Bioscience) using two or three color FACSCaliber analysis.

Statistical analysis

Statistical significance in the frequency of coronary lesions in the experimental groups was determined using Fisher's exact test and the severity of coronary lesions in the experimental groups were analyzed with the Mann-Whitney U test.

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Abbreviations

CAV	cardiac allograft vasculopathy
Tregs	regulatory T cells

References

1. Stehlik J, et al. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant*. 2011; 30(10):1078–1094. [PubMed: 21962016]
2. Weiss MJ, et al. Mechanisms of chronic rejection in cardiothoracic transplantation. *Front Biosci*. 2008; 13:2980–2988. [PubMed: 17981771]
3. Rahmani M, et al. Allograft vasculopathy versus atherosclerosis. *Circ Res*. 2006; 99(8):801–815. [PubMed: 17038650]
4. Russell PS, et al. Coronary atherosclerosis in transplanted mouse hearts. I. Time course and immunogenetic and immunopathological considerations. *Am J Pathol*. 1994; 144(2):260–274. [PubMed: 7906094]
5. Russell PS, et al. Coronary atherosclerosis in transplanted mouse hearts. II. Importance of humoral immunity. *J Immunol*. 1994; 152(10):5135–5141. [PubMed: 8176230]
6. Hirohashi T, et al. Complement independent antibody-mediated endarteritis and transplant arteriopathy in mice. *Am J Transplant*. 2010; 10(3):510–517. [PubMed: 20055805]
7. Kitchens WH, et al. Macrophage depletion suppresses cardiac allograft vasculopathy in mice. *Am J Transplant*. 2007; 7(12):2675–2682. [PubMed: 17924996]
8. Uehara S, et al. NK cells can trigger allograft vasculopathy: the role of hybrid resistance in solid organ allografts. *J Immunol*. 2005; 175(5):3424–3430. [PubMed: 16116237]
9. Hirohashi T, et al. A novel pathway of chronic allograft rejection mediated by NK cells and alloantibody. *Am J Transplant*. 2012; 12(2):313–321. [PubMed: 22070565]
10. Graham JA, et al. Viral infection induces de novo lesions of coronary allograft vasculopathy through a natural killer cell-dependent pathway. *Am J Transplant*. 2009; 9(11):2479–2484. [PubMed: 19843029]
11. Barao I, et al. Suppression of natural killer cell-mediated bone marrow cell rejection by CD4+CD25+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2006; 103(14):5460–5465. [PubMed: 16567639]
12. Ghiringhelli F, et al. The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. *Immunol Rev*. 2006; 214:229–238. [PubMed: 17100888]
13. Ghiringhelli F, et al. CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J Exp Med*. 2005; 202(8):1075–1085. [PubMed: 16230475]

14. Kamath AT, Sheasby CE, Tough DF. Dendritic cells and NK cells stimulate bystander T cell activation in response to TLR agonists through secretion of IFN-alpha beta and IFN-gamma. *J Immunol.* 2005; 174(2):767–776. [PubMed: 15634897]
15. Tanaka M, et al. Cardiomyocyte-specific Bcl-2 overexpression attenuates ischemia-reperfusion injury, immune response during acute rejection, and graft coronary artery disease. *Blood.* 2004; 104(12):3789–3796. [PubMed: 15280201]
16. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol.* 2007; 8(2):191–197. [PubMed: 17136045]
17. Russell PS, et al. Coronary atherosclerosis in transplanted mouse hearts. III. Effects of recipient treatment with a monoclonal antibody to interferon-gamma. *Transplantation.* 1994; 57(9):1367–1371. [PubMed: 7910422]
18. Tellides G, et al. Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature.* 2000; 403:207–211. [PubMed: 10646607]
19. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. *Nat Rev Immunol.* 2009; 9(7):480–490. [PubMed: 19543225]
20. Gasteiger G, et al. IL-2-dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells. *J Exp Med.* 2013; 210(3):1167–1178. [PubMed: 23650441]
21. Sitrin J, et al. Regulatory T cells control NK cells in an insulinitic lesion by depriving them of IL-2. *J Exp Med.* 2013; 210(6):1153–1165. [PubMed: 23650440]
22. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973; 16(4):343–350. [PubMed: 4583148]
23. McNeill A, Spittle E, Backstrom BT. Partial depletion of CD69^{low}-expressing natural regulatory T cells with the anti-CD25 monoclonal antibody PC61. *Scand J Immunol.* 2007; 65(1):63–69. [PubMed: 17212768]
24. Armstrong AT, et al. Morphometric analysis of neointimal formation in murine cardiac allografts: II. Rate and location of lesion development. *Transplantation.* 1997; 64(2):322–328. [PubMed: 9256195]
25. Walzer T, et al. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc Natl Acad Sci U S A.* 2007; 104(9):3384–3389. [PubMed: 17360655]

Figure 1. Elastin stain of proximal coronaries from parental to F1 allografts explanted at 3 weeks

A) An advanced, hypercellular CAV lesion present in allografts from recipients treated with anti-CD25 mAb. **B)** an early CAV lesion present in allografts from untreated recipients. **C)** no lesions were present in allografts from recipients treated with both anti-CD25 mAb and anti-NK1.1 mAb. Black arrows indicate the external elastic lamina. Original magnification x200.

Figure 2.

A: Morphometric analysis of CAV lesions in allografts from recipients treated with anti-CD25 mAb, with or without anti-NK1.1 mAb or anti-CD4 mAb and explanted at 3 weeks. The severity of CAV in the anti-CD25 mAb treatment group is significantly higher than in any other group (Mann Whitney U test). Anti-NK1.1 mAb completely blocked the effect of anti-CD25 mAb on lesion formation. Anti-CD4 had no effect by itself but inhibited the severity in CAV formation observed following anti-CD25 treatment. (* = $P < 0.05$; ** = $P < 0.005$; *** = $P < 0.01$; **** = $P < 0.0005$).

B: NK cell infiltrate in CAV lesions in allografts from untreated and anti-CD25 mAb treated recipients at 3 weeks. Cryostat sections of cardiac allografts stained with anti-NKp46 (NK cells) and a secondary antibody labeled with Cy5 (red). White arrows indicate internal elastic lamina of coronary arteries. Elastin autofluoresces green. **Control:** Coronary lesions in allografts from untreated recipients showed only scattered NK cells in the intima. **α-CD25:** Abundant NK cells were present in the intima and adventia of coronary lesions in allografts from recipient treated with anti-CD25mAb. Original magnifications x200.

Figure 3. Immunohistochemical analysis of CAV lesions in allografts from anti-CD25 mAb treated recipients at 3 weeks

Cryostat sections of allografts developed with diaminobenzidine (brown). Red arrows indicate the external elastic lamina. Black arrows indicate internal elastic lamina. Intimal regions are depicted by dashed line circle and the adventitial areas by a solid line circle. **a)** Numerous CD3⁺ cells are present in the coronary artery intima and adventitia. **b)** CD4⁺ cells are also present more outside of the coronary lesion. **c)** CD11c⁺ cells, probably dendritic cells, are more prominent in the adventitia than intima. **d)** CD16⁺ cells, present on NK cells and macrophages, are present in both the intima and adventitia. Original magnifications: a, b, c, and d x400.

Figure 4. Immunohistochemical analysis of CAV lesions in allografts from anti-CD25 mAb and anti-NK1.1 mAb treated recipients at 3 weeks

Co-treatment with anti-CD25 mAb and anti-NK1.1 mAb results in the absence of CD3+ and CD4+ cells in the lesions when compared to recipients treated with anti-CD25 alone. No Foxp3+ cells were observed in the untreated animals and in the lesions of recipients treated with anti-CD25 alone. Black arrows indicate stained cells. Original magnification x200.

Table 1

Allograft vasculopathy in parental to F1 heart transplants.

Group	Donor	Recipient	Explant Time (weeks)	Treatment	Hearts with CAV	p Value
1	B6	CB6F1	3	No Rx	6/8	vs. group 2 p<0.05 vs. group 7 p<0.01
2	B6	CB6F1	3	Anti-NK1.1	0/5	
3	B6	CB6F1	3	Anti-CD25	12/12	vs. group 7 p<0.0001 vs. group 1 N.S.
4	CB6F1	CB6F1	3	Anti-CD25	0/3	
5	B6	CB6F1	2	No Rx	0/3	
6	B6	CB6F1	2	Anti-CD25	6/7	vs. group 5 p<0.05
7	B6	CB6F1	3	Anti-CD25 & Anti-NK1.1	0/7	
8	B6	CB6F1	3	Anti-CD4	5/5	vs. group 3 N.S.
9	B6	CB6F1	3	Anti-CD25 & Anti-CD4	5/5	vs. group 3 N.S.