CD8+ T cells mediate aortic allograft vasculopathy by direct killing and an interferon-γ-dependent indirect pathway

Anton I. Skaroa,1, Robert S. Liwskib,1, Juan Zhoub, Ellen L. Vessieb, Timothy D.G. Leea,b,*, Gregory M. Hirscha

aDepartment of Surgery, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7
bDepartment of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Abstract

Objective: Allograft vasculopathy (AV) has emerged as the major obstacle to long-term survival in clinical heart transplantation. Immune events are implicated in the development of AV, but the cellular and molecular mechanisms involved remain unclear. We sought to determine whether and by what mechanism CD8+ T lymphocytes are able to generate AV in a murine aortic allograft model.

Methods: Allo-primed CD8+ T lymphocytes were transferred into immunodeficient (RAG-1−/−) mouse recipients of aortic allografts. We also transferred primed CD8+ T cells with targeted deletions of effector molecules (perforin, Fas-ligand) to determine the role of direct cytolysis (CTL) in CD8+ T-cell-mediated AV. We determined the role of non-CTL effector mechanisms through the transfer of either wildtype or interferon-γ (IFN-γ)-deficient CD8+ T cells into RAG-1−/− recipients of MHC class I-deficient allografts.

Results: Adoptive transfer of primed wildtype CD8+ T lymphocytes into immunodeficient recipients of aortic allografts resulted in the development of robust AV lesions. Transfer of CD8+ T lymphocytes with targeted deletions in CTL effector molecules resulted in reduction of AV lesion size but not abrogation. Transfer of wildtype CD8+ T cells into recipients of MHC class I-deficient grafts resulted in a reduction in AV lesion size, while transfer of interferon-γ-deficient CD8+ T cells into MHC class I-deficient grafts abrogated AV.

Conclusions: These data indicate that CD8+ T cells mediate AV through direct cytolysis and a distinct interferon-γ-dependent non-CTL effector pathway. Given the resistance of this cell type to conventional immunosuppression, these results may have important therapeutic implications.

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1. Introduction

Late cardiac graft loss due to allograft vasculopathy (AV) remains a major limitation in clinical heart transplantation [1]. AV consists of a concentric thickening of the intimal layer of the epicardial coronary arteries, leading to luminal occlusion, thrombosis and eventually ischemic organ failure [2]. In addition, AV is characterized by the presence of an immune infiltrate composed of lymphocytes and mononuclear cells in the adventitia and the loss of smooth muscle cells (SMC) from the media [3]. Current immunosuppressive regimens do not inhibit AV and conventional revascularization strategies are ineffective because of the diffuse nature of the disease [1].

The cellular and molecular mechanisms involved in the generation of AV are not known. It is known that AV is a T-cell-mediated phenomenon, in that athymic nude animals do not exhibit AV [4], although recent evidence suggests that
innate immunity may also contribute to lesion formation [5]. While some have suggested that CD4+ T cells are necessary for the generation of AV [4,6], others have provided evidence that the development of AV is less dependent than acute rejection on CD4+ T-cell helper function. For example, AV occurs in anti-CD4 antibody transgenic mice that are devoid of CD4+ T cells by endogenous depletion [7]. We have shown that marked apoptotic cell death occurs within the medial compartment during early stages of AV, with concomitant up-regulation of mRNA for cytotoxic T-lymphocyte-derived mediators of target cell apoptosis [3]. Taken together, these data suggest an important role for CD8+ T cells in the development of AV.

In the experimentation reported here, we explored the role of primed CD8+ T cells in the development of AV using the well established aortic interposition allograft model. This model was chosen for consistency of lesion formation and the ease with which these lesions can be quantified [8]. Ensminger et al. [9] have shown that the degree of intimal hyperplasia is highly concordant between aortic allografts and whole organ transplants in a combined heterotopic heart and aortic allograft model. A similar correlation was found in allograft aortas and coronary arteries of human transplants undergoing AV [10]. Furthermore, we [11,12] and others [13] have found that immunosuppression using cyclosporine A, which ameliorates acute rejection in this model, does not affect the generation of AV. In fact, data from both immunosuppressed [11,13] and non-immunosuppressed [11,14] animal models including aortic allografts [11,13,14] and whole organ transplants [13–15] have conclusively demonstrated that recipient-derived cells form the neointimal lesion (reviewed in Ref. [16]). These observations, which were made in the aortic allograft model, have been pivotal in revising the previously accepted hypothesis regarding the etiology of AV [11,13,14].

Our data demonstrate that CD8+ T cells induce AV in the absence of CD4+ T cells or antibody, and that CTL-mediated direct killing and interferon-γ (IFN-γ)-dependent non-CTL effector pathways are both involved. Given that CD8+ T cells [17,18] and indirect allo-recognition [19] have been shown to be relatively resistant to standard, clinical immunosuppressive agents, these data might explain the limited efficacy of current immunosuppressive therapy in preventing AV.

2. Materials and methods

2.1. Animals

Male, 6-week-old, BALB/cJ (BALB/c; H-2b), C3H/HeJ (C3H; H-2k), C3.MRL-Tnfrsf5+tm1 (lpr; H-2b), C57BL/6 (B6; H-2b), C57BL/6J-B2m-1Unc (B6 β2m2−; H-2b), MRL/MpJ (MRL; H-2b) and MRL.129P2(B6)-B2m1Unc (MRL β2m2−; H-2b) mice were used as donors. Male, 8-week-old, B6.129S7-RAG-1tm1Mom (B6 RAG-1−/−; H-2b), B6.129S2Cd8am1Mak (B6 CD8−−; H-2b) and C3H.129S6(B6)-Rag2am1 N12 (C3H RAG-2−/−; H-2b) mice were used as recipients. Male, 6-week-old, B6, C3H, C57BL/6-Pip5tmcdn (pp6; H-2b), B6Smn.C3-Tnfsf8g0d (gld; H-2b) and B6.129S7-lfng (IFN-γ−/−; H-2b) were used as cell donors for adoptive transfer and in vitro culture. In a separate experiment, C3H/HeJ (H-2b) donor aortas were transplanted into either C3H.129S6(B6)-Rag2am1 gld (referred to as CD8−/−) with and without cyclosporine (CyA) immunosuppression (see below). All mice were purchased from Jackson Laboratories (Bar Harbor, ME) or Taconic Farms (Germantown, NY) and maintained in the Carleton Animal Care Facility with food and water ad libitum. Sentinel mice were serologically negative for all viral pathogens. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council on Animal Care and conforms to NIH guidelines.

2.2. Aortic transplantation

Abdominal aortas were transplanted as previously described [8]. Briefly, a 10-mm segment of BALB/c or C3H abdominal aorta was transplanted orthotopically into a B6 RAG-1−/− or B6 CD8−/− recipient mouse, respectively. Graft and spleen harvest, including serum sampling, was performed at 60 days post-transplantation.

2.3. Allo-immunization

Mitomycin C (25μg/ml)-inactivated BALB/c spleenocytes were used for in vivo allo-immunization of B6 cell donors. Groups of 6-week-old male B6 mice (4 mice/group) were immunized intraperitoneally with 5×107 mitomycin C-treated BALB/c spleenocytes in 200 μl of PBS.

2.4. Whole T cells and CD8+ T-cell isolation

On day 7 following allo-immunization, single cell suspensions from spleens of B6 mice were prepared as described previously [20] Whole T cells were enriched by nylon wool columns (Polysciences, Warrington, PA) [20,21] followed by passage through CD3 immunocolumns (R&D Systems, Minneapolis, MN). For CD8+ T-cell isolation, whole T-cell suspensions were purified by negative selection with CD8 immunocolumns (R&D Systems) [21]. CD8 purity was >93% by flow cytometry with no detectable CD4+ or B220+ contaminants [21,22]. For the isolation of non-CD8+ cells, splenocytes were treated with anti-CD8 mAb (0.2 μg/106 cells; clone YTS169.4; Cedarlane, Mississauga, ON) and low-tox-complement (Cedarlane) prior to nylon wool enrichment and passage through CD8 immunocolumns.

2.5. Direct cytotoxic T-lymphocyte (dCTL) activity

Ex vivo allo-specific dCTL activity was assessed to confirm allo-activation of primed T-cell populations.
Briefly, freshly purified, primed B6 whole T and CD8\(^+\) T cells were cultured in the presence of \(^{3}\text{H}\)thymidine (\(^{3}\text{H}\)TdR)-labeled allogeneic P815 (H-2\(^d\); ATCC) or third-party BW5147.3 (H-2\(^k\); ATCC) tumor cell targets at effector to target cell ratios ranging from 50:1 to 6.25:1. The plates were incubated for 18 h at 37 °C and after harvesting apoptosis was calculated based on the loss of \(^{3}\text{H}\) due to DNA fragmentation [20]. To assess direct killing in the absence of the granule–exocytosis and Fas–Fas ligand pathways, primed, purified CD8\(^+\) T cells from B6 perforin-deficient mice, expanded in vitro for 72 h, were prepared as effector cells and \(^{3}\text{H}\)Tdr-labeled allogeneic C3H lpr concanavalin A-stimulated splenic lymphoblasts were used as target cells. Cells were plated at effector to target ratios ranging from 25:1 to 3:1. After incubation at 37 °C for 4 h, the plates were harvested and specific apoptosis was calculated as described above.

2.6. Adoptive cell transfer

Twenty-four or ninety-six hours after transplantation, B6 RAG-1\(^{-/-}\) recipients were injected intraperitoneally with 1\( \times 10^7\) purified whole T cells or CD8\(^+\) T cells isolated from allo-immunized B6 mice.

2.7. Immunosuppression

B6 and B6 CD8\(^{-/-}\) aortic allograft recipients that were treated with immunosuppression received either 50 or 70 mg/kg/day of CyA (Neoral), diluted in sterile saline, subcutaneously for 8 weeks.

2.8. Histology and morphometry

Formalin-fixed and paraffin-embedded tissues were used for conventional histology. Eight sections from each graft were stained with Verhoeff elastin stain. Digitized images were captured using a Zeiss Axiovert 200 and AxioCam camera (Carl Zeiss, Thornwood, NY) and the intimal and medial areas were measured using Scion Image software (Scion, Frederick, MD) [3,12].

2.9. Immunocytochemistry

Frozen sections (6 \(\mu\)m) were stained with the following antibodies: anti-CD3e (145-2C11; 1:50), anti-CD4 (RM4-5; 1:25), anti-CD8a (53-6.7; 1:50), anti-CD11b (M1/70; 1:50) and anti-CD19 (1D3; 1:25) purchased from PharMingen (SanDiego, CA). Primary antibody was detected with biotinylated anti-hamster IgG (G70-204, G94-56; 1:50, PharMingen) for CD3 and polyclonal anti-rat IgG antibody (1:50; PharMingen) for CD4, CD8, CD11b and CD19 followed by treatment with peroxidase avidin/biotin complex (Vector, Burlingame, CA), with 3,3′ diaminobenzidine as the chromagen. As a negative control, species and IgG isotype matched antibodies were used in place of primary antibody (PharMingen). All incubations were for 1 h at 25 °C.

2.10. Statistics

Data are presented as mean±SEM for each experimental group. One-way ANOVA and Tukey–Kramer multiple comparisons test, Student’s t-test and Mann–Whitney tests were used. Values of \(p<0.05\) were considered significant.

3. Results

3.1. Generation of primed effector CD8\(^+\) T cells

In this study, we utilize adoptive cell transfer into immunodeficient RAG-1\(^{-/-}\) recipients that lack functional B-cell and T-cell compartments to examine the contribution of CD8\(^+\) T cells to the generation of AV. We restricted our observations to the effector limb, by allowing primary antigen activation to occur in the wildtype host and subsequently transferring effector T-cell populations.

To confirm priming, we tested freshly purified, primed CD8\(^+\) T cells for direct CTL activity ex vivo. Primed CD8\(^+\) T cells from allo-immunized animals effectively killed allogeneic (H-2\(^d\)) targets, whereas naive CD8\(^+\) T cells did not (Fig. 1). Allo-primed CD8\(^+\) T cells did not kill third-party (H-2\(^k\)) targets. Deletion of CD8\(^+\) cells from primed whole T-cell populations eliminated killing (data not shown).

![Fig. 1. Generation of primed allo-specific CD8\(^+\) CTL. CD8\(^+\) T cells, freshly purified from allo-primed animals, were tested for killing activity in an ex vivo direct CTL assay. Allogeneic targets were incubated with naive CD8\(^+\) T cells (•) or primed CD8\(^+\) T cells (■). Third party targets were also used (△, □). Experiment shown is representative of seven separate experiments (**p<0.001, ANOVA).](https://academic.oup.com/cardiovascres/article-abstract/65/1/283/310142/fig1)
3.2. Role of CD8\(^+\) effector T cells in the generation of AV

After demonstrating that allo-primed CD8\(^+\) T cells are effective killers in vitro, we assessed their ability to generate AV in B6 RAG-1\(^{-/-}\) recipients of fully mismatched BALB/c aortic allografts. Grafts were placed into: unreconstituted B6 RAG-1\(^{-/-}\) recipients, whole T-cell-reconstituted B6 RAG-1\(^{-/-}\) recipients and wildtype recipients (as controls). As expected, there was no AV in unreconstituted RAG-1\(^{-/-}\) mice (Fig. 2), whereas reconstitution with primed whole T cells or with purified, primed CD8\(^+\) T cells (Fig. 2) led to the generation of robust AV. These mice showed a marked intimal lesion, virtually complete loss of medial SMC and a profound adventitial mononuclear cell infiltrate. When CD8\(^+\) cells were deleted from the purified CD8\(^+\) T-cell transfer population, by antibody and complement-mediated lysis, there was complete abrogation of AV (Fig. 2).

Intimal area was calculated by digital morphometry. Wildtype recipients, RAG-1\(^{-/-}\) recipients reconstituted with primed whole T cells and RAG-1\(^{-/-}\) recipients reconstituted with primed CD8\(^+\) T cells all developed intimal hyperplasia at similar levels (Fig. 2). Medial area calculations revealed a statistically significant reduction for CD8\(^+\) T-cell-reconstituted, whole T-cell-reconstituted and wildtype animals, when compared with unreconstituted Rag1\(^{-/-}\) animals as a control (CD8\(^+\) T-cell-reconstituted: 90,553 \(\pm\) 6066 \(\mu\)m\(^2\)), 36.4% reduction) (whole T-cell-reconstituted: 84,333 \(\pm\) 7131 \(\mu\)m\(^2\)), 40.7% reduction) (wildtype unreconstituted: 72,194 \(\pm\) 7986 \(\mu\)m\(^2\)), 49.2% reduction) (unreconstituted Rag1\(^{-/-}\) control 142,319 \(\pm\) 4523 \(\mu\)m\(^2\)) (all reductions \(p<0.001\)).

Cellular infiltration into the allografts was assessed by immunocytochemistry. As expected, allografts harvested from unreconstituted RAG-1\(^{-/-}\) recipients did not contain CD4\(^+\) or CD8\(^+\) cells (Fig. 3). Allografts harvested from RAG-1\(^{-/-}\) mice reconstituted with whole T cells showed CD4\(^+\) T cells mainly within the adventitia with CD8\(^+\) T cells primarily in the media (Fig. 3). Allografts harvested from RAG-1\(^{-/-}\) mice reconstituted with primed effector CD8\(^+\) T cells showed CD8\(^+\) T cells localized predominantly within the media and a small number in the intimal compartment, with no CD4\(^+\) T cells in the grafts (Fig. 3). These data confirm that transferred effector CD8\(^+\) T cells migrate to the graft and mediate AV in the absence of CD4\(^+\) T cells or B cells.

3.3. CD8\(^+\) T-cell-mediated direct killing contributes to the generation of AV

We examined the role of direct cytolytic and non-cytolytic effector mechanisms in CD8\(^+\) T-cell-mediated AV. We first assessed direct killing in vitro by using primed CD8\(^+\) T cells with targeted deletions of effector molecules necessary for the granule–exocytosis and the Fas–Fas ligand (FasL) pathways of direct killing.

As expected, primed wildtype CD8\(^+\) T cells induced substantial killing of allogeneic targets in vitro (Fig. 4). When the granule–exocytosis pathway was interrupted, by using perforin deficient (\(pfp\)) effector cells, killing was not reduced (Fig. 4). Likewise, interruption of the Fas–FasL pathway, by using Fas receptor defective (\(lpr\)) target cells, did not reduce killing (Fig. 4). However, when both pathways were interrupted, by using \(pfp\) effector cells against \(lpr\) targets, complete abrogation of killing activity was observed (Fig. 4). These data confirm that, at least in vitro, minor pathways of direct killing are not relevant in this system.

We subsequently used these mouse combinations for in vivo experimentation. When primed CD8\(^+\) T cells from B6 FasL-deficient (\(gld\)) mice or B6 perforin-deficient (\(pfp\)) mice were used to reconstitute B6 RAG-1\(^{-/-}\) allograft...
recipients, we observed robust AV (Fig. 5), confirming that neither CTL pathway is essential for the generation of AV. When we blocked both of the major pathways of direct killing simultaneously by transferring primed CD8+ T cells derived from pfp animals into RAG-1/C0/C0 recipients transplanted with lpr aortic allografts, the mice exhibited attenuated AV lesions. The AV lesion was characterized by both intimal hyperplasia and medial SMC loss (Fig. 5), but there was an almost 50% reduction in intimal hyperplasia compared to wildtype controls (Fig. 5, p<0.01) suggesting an important, but not essential, role for direct killing in the etiology of AV, as well as the activity of some alternate non-CTL CD8 effector pathway.

3.4. A distinct CD8 non-CTL effector pathway is capable of generating AV and is interferon-γ-dependent

To examine the contribution of non-CTL effector mechanisms, we transferred purified, primed C3H CD8+ T cells into immunodeficient C3H RAG-2/C0/C0 recipients transplanted with lpr aortic allografts. These grafts express undetectable levels of surface Class I MHC. We (Fig. 6) and others [23–26] have shown that such
cells are not susceptible to direct killing. The cells do, however, produce class I peptides, which can be presented by recipient dendritic cells and used to engage the transferred effector CD8+ T cells. Such CD8+ T cells may then mediate killing by indirect non-CTL means. These grafts exhibited AV, but the extent of intimal hyperplasia was reduced by about one third when compared to wildtype control allografts (Fig. 7, \( p = 0.0286 \)). These data implicate non-cytolytic effector pathways in CD8+ T-cell-mediated AV.

Having implicated non-cytolytic CD8+ T-cell effectors in the generation of AV, we postulated that IFN-\( \gamma \) would be a prime mediator of this pathway. To confirm both the indirect non-CTL effect and a role for IFN-\( \gamma \) in this pathway, we used primed CD8+ T cells from IFN-\( \gamma \)/−/− mice to reconstitute RAG-1−/− recipients of \( \beta_{2m} \)-/− allografts. Primed CD8+ T cells from IFN-\( \gamma \)/−/− mice were unable to generate AV in these allografts despite a significant CD8+ T-cell infiltrate (Fig. 8). These data confirm that a non-CTL pathway is involved in the observed AV in \( \beta_{2m} \)-/− allografts and that IFN-\( \gamma \) is the essential mediator of this process.

3.5. CyA prevents AV in CD8−/− but not wildtype recipients of aortic allografts

Our findings that CD8+ T cells are capable of mediating AV in the absence of CD4 help may explain the inability of CyA to inhibit AV. Recent studies indicate CD8+ T cells are relatively resistant to the effects of CyA. To test this hypothesis C3H aortas were transplanted into B6 wildtype recipients of B6 wildtype CTL, primed by allo-immunization with wildtype MRL (\( \beta_{2m} \)-/+) cells, were incubated in a 4-h in vitro CTL assay with wildtype MRL ConA lymphoblast targets (a) or with MRL \( \beta_{2m} \)-/− ConA lymphoblast targets (b). Third party control targets were also tested and no killing was observed. Non-specific target lysis was <7%.

**Fig. 5. Contribution of CD8+ T cell-mediated direct killing to AV.** Wildtype allografts were transplanted into RAG-1−/− mice which received primed CD8+ T cells from either wildtype mice (a), FasL−/− mice (b) or from perforin-deficient mice (c). These combinations block either the Fas–FasL pathway (b) or the granule–exocytosis pathway (c) of killing. Also examined were allografts from Fas−/− mice transplanted into RAG-1−/− mice reconstituted with primed CD8+ T cells from perforin-deficient mice (d) (blocks both pathways). Micrographs are representative of \( n=5–6 \) in all groups. Digital image analysis of sections yielded quantitative data of the extent of intimal hyperplasia in the five groups: \( n=5–6 \) in all groups (c) (**\( p<0.01 \), ANOVA).

**Fig. 6. Ablation of CTL activity against \( \beta_{2m} \)-deficient targets.** Wildtype B6 CTL, primed by allo-immunization with wildtype MRL (\( \beta_{2m} \)-/+) cells, were incubated in a 4-h in vitro CTL assay with wildtype MRL ConA lymphoblast targets (a) or with MRL \( \beta_{2m} \)-/− ConA lymphoblast targets (b). Third party control targets were also tested and no killing was observed. Non-specific target lysis was <7%.

**Fig. 7. Contribution of indirect effectors to CD8+ T-cell-mediated AV.** Allografts from wildtype B6 (a) or B6 \( \beta_{2m} \)-/− (little or no surface expression of class I MHC) (b) mice were transplanted into C3H RAG-2−/− recipients receiving primed, purified CD8+ T cells from wildtype C3H mice. AV in \( \beta_{2m} \)-/− grafts is due to indirect CD8+ T-cell-mediated effects. Digital image analysis of sections (c) quantified the intimal area in these groups; \( n=4 \) for both groups (**\( p=0.0286 \), Mann–Whitney).
or B6 CD8<sup>-/-</sup> recipients treated with CyA. At 50 mg/kg/day of CyA, CD8-deficient recipients did not undergo AV lesion formation, histology at 8 weeks in these animals revealed a normal media and no intimal lesion formation. In contrast, wildtype recipients, at both 50 and 70 mg/kg/day of CyA, underwent both medial loss and intimal lesion formation characteristic of AV (Fig. 9).

**4. Discussion**

Although many factors (both immunologic and non-immunologic) contribute to the generation of AV [17,27], much research has established that in the absence of effective T-cell activity AV does not occur [4,28–30]. In this study, T- and B-cell-deficient RAG-1<sup>-/-</sup> mice did not exhibit AV.

Some have suggested that CD4<sup>+</sup> T helper cells are necessary for the generation of AV [4,6,31]. However, we using CD4 knockout mice (manuscript submitted) and others, using endogenous antibody depletion [7], have found that AV is not dependent on the activities of CD4<sup>+</sup> T cells. Here, we demonstrate that primed CD8<sup>+</sup> T cells are sufficient to generate AV. Transferred purified effector CD8<sup>+</sup> T cells induced robust AV in RAG-1<sup>-/-</sup> mice, which lack both T cells and B cells. When effector CD8<sup>+</sup> cells were deleted from the transfer population by antibody depletion, AV induction was abolished, confirming that the AV lesions seen are dependant on effector CD8<sup>+</sup> T cells. The transferred CD8<sup>+</sup> T cells trafficked largely to the media, with a small number in the intimal compartment, suggesting the possibility of a role in both medial and intimal injury as a potential inciting stimulus for the development of AV.

CD8<sup>+</sup> T cells are capable of direct killing [3,20], but they also produce cytokines which mediate DTH-like immune responses [21,22]. We first examined the role of direct CTL-mediated killing. When either the granule exocytosis or Fas–FasL pathways were blocked independently, AV lesion formation was largely unimpeded. These data are in contrast to the observations of Subbotin et al. [32], who claimed that Fas–FasL interactions were critical for the development of AV. This disparity may be explained by the fact that...
Subbotin’s group used older mice, given that gld mice demonstrate age dependent immunological hyporesponsiveness [33].

When both pathways of direct killing were blocked concurrently, we observed a significant attenuation of CD8⁺ T-cell-mediated AV indicating that direct killing does play a role in the generation of AV. However, since complete inhibition of AV was not seen, other pathways must be active. Having demonstrated in vitro that simultaneous blockade of granule–exocytosis and Fas–FasL results in complete inhibition of direct killing, it is unlikely that minor pathways of direct killing are active within this system. Since neither unreconstituted recipients, nor animals in which CD8⁺ cells were deleted from the transfer population, developed lesions, NK cells of the host cannot be directly implicated. Taken together, these data indicate that non-CTL effector mechanisms contribute to CD8⁺ T-cell-mediated AV.

We assessed the non-cytolytic CD8⁺ T-cell effector pathway by evaluating the ability of allo-primed CD8⁺ T cells to reject β2m−/− grafts. In these grafts, surface MHC-I expression is virtually undetectable and CD8⁺ T cells are unable to directly interact with such grafts [23,26,34]. Primed CD8⁺ T cells generated AV within β2m−/− allografts despite the mitigation of direct killing. Although there is residual MHC class I expression in these animals [24–26], the functional status of these free heavy chains is questionable [23,25,26]. Moreover, primed CD8⁺ T cells generated by immunization with allogeneic wildtype (β2m+/+) cells do not kill β2m−/− targets in vitro (Fig. 6) [25]. Given these data, we conclude that indirect effectors are involved in the generation of AV. Such indirect effector CD8 function is implicated by the recent findings of He and Heeger [35] who demonstrated that B6 D2Kb−/− skin allografts (no MHC class I expression) could be rejected by C3H SCID mice reconstituted with CD8⁺ T lymphocytes.

IFN-γ would be a likely mediator of this non-CTL effector mechanism [36]. For example, in mouse cardiac allografts, CD8⁺ T lymphocytes have been identified as an abundant source of IFN-γ [37], and Valujskikh et al. [38] have demonstrated the presence of indirectly primed graft infiltrating CD8⁺ T cells capable of allo-specific production of IFN-γ. IFN-γ could potentiate PDGF-induced effects on the cells forming the neointimal lesion [39] as well as inducing DTH-like cell-mediated responses [40]. In this study, transfer of allo-primed CD8⁺ T cells from IFN-γ−/− mice into RAG-1−/− recipients where the direct CTL effector pathways are blocked did not induce AV, demonstrating that the observed non-CTL effector pathway is IFN-γ-dependent.

The ability of CD8⁺ effector T cells to generate the AV lesion in the absence of CD4⁺ T-cell help could explain the resistance of AV to current immunosuppressive treatments, which primarily target CD4⁺ T-cell activation [17,18,41]. Strehlau et al. [42], for example, have demonstrated significant CD8⁺ T-cell activity in the clinical setting despite immunosuppression. This is likely explained by sensitivity of CD4⁺ T cells, but relative resistance of CD8⁺ T cells, to calcineurin inhibitors [17,18]. This correlates with our evidence [11,12] and the evidence of others [13] that in animal models moderate calcineurin inhibition eliminates CD4⁺ T-cell-mediated acute rejection but has little to no effect on AV.

To determine whether CD8⁺ T cells are indeed responsible for CyA resistance with regard to the generation of AV, we administered a dose of CyA sufficient to block acute rejection in a murine cardiac allograft model (50 mg/kg/day). Aortic allografts from wildtype animals treated with this dose for 8 weeks went on to form classic AV lesions with both intimal hyperplasia and medial destruction. In contrast, CD8 deficient animals were free of AV. When we increased the dose to 70 mg/kg/day, the results did not change, with wildtype animals still exhibiting AV (Fig. 9). Thus, the AV observed in immunosuppressed mice is dependent upon CD8⁺ T-cell effector function. The activity of non-CTL effector CD8⁺ T cells might be particularly implicated in the failure of modern transplant pharmacotherapy, since indirect pathways in general are thought to be most resistant to calcineurin inhibitors [19,43]. These data from the murine aortic allograft model suggest novel avenues for exploration of human AV, which may result in effective therapeutic approaches to the prevention of human AV.

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