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Acute Cardiac Rejection Requires Directly Cytotoxic CD4 T cells: A Parallel Pathway between Fas and Perforin¹

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Abstract

Background—CD4 T cells can suffice as effector cells to mediate primary acute cardiac allograft rejection. While CD4 T cells can readily kill appropriate target cells in vitro, the corresponding role of such cytolytic activity for mediating allograft rejection in vivo is unknown. Therefore, we determined whether the cytolytic effector molecules perforin and/or FasL (CD95L) were necessary for CD4 T cell-mediated rejection in vivo.

Methods—Wild type C3H(H-2^k) or Fas (CD95)-deficient C3H/*lpr* (H-2^k) hearts were transplanted into immune-deficient C57B6*rag*^{−/−} (H-2^b) mice. Recipients then were reconstituted with naïve purified CD4 T cells from either wild-type, perforin (*pfp*)-deficient, or FasL (*gld*)-deficient T cell donors.

Results—In vitro, alloreactive CD4 T cells were competent to lyse donor MHC class II+ target cells, largely by a Fas-dependent mechanism. In vivo, the individual disruption of either donor Fas expression (*lpr*) or CD4 T cell-derived perforin had no significant impact on acute rejection. However, FasL-deficient (*gld*) CD4 T cells demonstrated delayed allograft rejection. Importantly, the simultaneous removal of both donor Fas expression and CD4 T cell perforin completely abrogated acute rejection, despite the persistence of CD4 T cells within the graft.

Conclusions—Results demonstrate that the direct rejection of cardiac allografts by CD4 effector T cells requires the alternative contribution of graft Fas expression and T cell perforin expression.

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To our knowledge, this is the first demonstration that cytolytic activity by CD4 T cells can play an obligate role for primary acute allograft rejection *in vivo*.

Keywords

Cytotoxicity; Cytotoxic CD4 T cells; Transplantation; Acute Rejection

Introduction

Chronic rejection is appreciated as the major source of cardiac allograft loss (1). However, this focus has arguably left the study of acute rejection under-examined. Importantly, acute rejection events have been consistently associated with the early development of graft vasculopathy (chronic rejection) and poor patient survival (1). Despite the use of nonspecific combined immunosuppressive agents, 50–80% of cardiac transplants suffer an episode of acute rejection in the first year (2,3). Therefore, identifying and abrogating effector pathways involved in acute rejection is important both for short- and long-term allograft survival, possibly by delaying the onset of graft dysfunction due to chronic rejection. Clearly, a variety of cellular and humoral effector pathways may contribute to allograft injury, involving both ‘direct’ (donor APC-dependent) and ‘indirect’ (host APC-dependent) donor antigen recognition. The specific goal of the current study was to determine the relative contribution of perforin- and Fas/FasL-dependent cytotoxic activity as potential mediators of CD4 T cell-mediated acute rejection *in vivo*.

It is well known that CD4 T cells are necessary for acute cardiac allograft rejection (4–7). Moreover, we previously found that CD4 T cells could be sufficient to serve as effector cells, independent of CD8 T cells or B cells, to mediate primary acute rejection (7,8). Importantly, CD4 T cell-mediated rejection required donor but not host MHC class II expression (7), possibly via direct recognition of allograft endothelial cells (8). However, it is unclear how CD4 T cells directly inflict this form of allograft injury. A potential, and arguably under-explored mechanism of CD4 T cell-mediated rejection may involve the Fas ligand (CD95L) and perforin (PFP) dependent pathways of direct cell-mediated cytotoxicity. The TNF family member FasL is a cell-surface molecule expressed on activated T cells and triggers apoptosis in target cells expressing the corresponding Fas (CD95) ligand (9,10). CD95/CD95L interaction is a primary effector pathway in T cell-mediated cytotoxicity (11,12) and often is the major pathway utilized by cytotoxic CD4 T cells (13,14). However, *in vivo* results indicate that donor Fas expression is not required for acute rejection, indicating that alternative effector mechanisms are sufficient for mediating graft injury (15,16). Alternatively, perforin/granzyme-dependent cytotoxicity is strongly associated with graft rejection (17–19) and generally is associated with CD8 T cell-dependent killing. While such pathways are not usually associated with CD4 T cell killing, CD4 T cells are indeed capable mediating target cell lysis via perforin *in vitro* in some cases (20). Examples of CD4 T cell cytotoxic activity in host defense are illustrated by studies showing the direct killing of Epstein-Barr virus (21) and lymphocytic choriomeningitis virus infected cells by cytotoxic CD4 T cells *in vivo* (22). Thus, the contribution of cytotoxic CD4 T cells to immune reactivity *in vivo* may be greater than previously thought.

In the current study, our primary objective was to determine the requirements for acute, contact-dependent CD4 T cell mediated cardiac allograft rejection *in vivo*. Due to prior studies suggesting a primary role for Fas/FasL CD4 T cell killing, we hypothesized that *in vivo* CD4 T cell mediated rejection would require donor Fas expression and would not require CD4 T cell perforin expression. Results show that removal of Fas from the donor heart or removal of perforin from the naive CD4 T cells does not abrogate rejection individually, but that removal of donor Fas and CD4 T cell perforin simultaneously completely abrogates rejection, despite

the accumulation of effector CD4 T cells within the allograft itself. These findings demonstrate that *in vivo* CD4 T cell-mediated cardiac rejection is dependent on donor Fas and CD4 T cell perforin expression in a parallel fashion and that a directly cytotoxic CD4 T cell is required for rejection to occur.

Materials and Methods

Mice

Inbred female C3H/HeJ (C3H, H-2^k) and Fas-deficient C3.MRL-*Tnfrsf6^{lpr}* (C3H *lpr*, H-2^k) (23) mice were utilized as heart donors and as a source of APCs for *in vitro* MLR assays. Inbred female BALB/cByJ (BALB/c, H-2^d) were used as a source for stimulator splenocytes for CTL assays. Inbred female C57B6ByJ (B6, H-2^b), perforin-deficient C57B6-*Prf1^{tm1Sdz}*/J (B6 PFPKO, H-2^b), and Fas Ligand-deficient B6Smn.C3-*Faslg^{ld}*/J (B6 *gld*, H-2^b) mice were used as lymph node donors for the column enrichment of CD4 T cells. Inbred female immune deficient C57B6-Rag1^{tm1/Mom} (B6 *rag*^{-/-}, H-2^b) mice were utilized as heart allograft recipients. All mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Animals were housed under pathogen-free conditions at the University of Colorado Barbara Davis Center Animal Facility and the University of Colorado Health Sciences Center Center for Comparative Medicine, according to NIH guidelines. All studies were reviewed and approved by the University of Colorado Institutional Animal Care and Use Committee.

Heterotopic heart transplantation

Cardiac allografts from C3H or C3H *lpr* donor mice were transplanted heterotopically into B6 *rag*^{-/-} recipient mice. Vascularized grafts were transplanted according to standard microsurgical techniques (24). Allograft survival was assessed by daily palpation with rejection defined as loss of palpable beating. Rejection was confirmed by direct visual inspection via laparotomy under anesthesia. Survival differences were determined using the Kaplan Meier Log Rank Test.

CD4 T cell purification and adoptive transfer

Cervical, axillary, epitrochlear, peri-aortic, and mesenteric lymph nodes (LNs) were harvested from B6, B6 PFPKO, or B6 *gld* mice. Single-cell suspensions of LN cells were enriched for CD4T cells by negative selection of CD8+ T cells and B-cells on an immunoaffinity column according to the manufacturer's specifications (Collect, Cedarlane, CL111-2, Ontario, Canada). Cellular phenotyping of freshly purified cells were determined by flow cytometry assessing staining of PE-labeled anti-CD4, anti-CD8+, and anti-CD19+ monoclonal antibodies (mAb) (PharMingen, San Diego, California, U.S.A.). B6 and B6 *pfp*^{-/-} CD4-enriched T cells contained less than 0.5% contaminating CD8+ T cells or CD19+ cells, whereas B6 *gld* CD4-enriched T cells contained less than 0.5% contaminating CD8+ T cells and less than 3% CD19+ cells (due to the larger number of CD19+ cells in *gld* mice pre-purification). Ten million CD4-enriched T cells were injected intraperitoneally into the indicated adoptive transfer recipients between day 3 and 5 post cardiac transplantation. As an added precaution, B6 *rag*^{-/-} cardiac transplant recipients were treated with rat anti-mouse CD8+ mAb 2.43 (IgG2b) (25) on post CD4 cell transfer day 0 and 7 for depletion of any CD8+ T cells that may have been transferred with the allograft/enriched cells. Anti-CD8+ mAb (2.43) was generated as ascites in *rag*^{-/-} mice and quantitated by isotype-specific ELISA.

Mixed lymphocyte reactions

Mixed lymphocyte reactions (MLR) of C3H, C3H *lpr*, or BALB/c splenocyte-stimulator cells with naïve B6, naïve B6 PFPKO, or naïve B6 *gld* CD4 T cells were performed. Briefly, quadruplicate wells containing 2.0×10^5 responder cells were mixed with 3.0×10^5 irradiated

(2500 Rads) splenic stimulator cells in 96-well flat bottom plates. Cells, cultured in EMEM supplemented with 10% FCS, 10⁻⁵ M 2-Me, and antibiotics, were incubated at 37°C in 10% CO₂. Cultures were then pulsed with 1.0 µCi thymidine for 6 hours on the indicated day of cell culture. Plates were harvested and counted on a Trilux 1450 micro beta scintillation counter (Wallac, Inc., Gaithersburg, Maryland, U.S.A.).

Cytotoxic T-lymphocyte assays

Cytotoxic T lymphocyte (CTL) activity *in vitro* was assessed by a standard ⁵¹Cr-release assay. Primary mixed-lymphocyte cultures, (naïve B6, B6 PFPKO, or B6 *gld* column-purified CD4 T cell effectors and BALB/c γ-irradiated splenocyte stimulators) were established in 24-well plates. On the fifth day of culture, effector CD4 T cells were harvested from these primary cultures and incubated in serial dilution with 1 × 10⁴ ⁵¹Cr-labeled BALB/c-derived A20 B-cell lymphoma target T cells (26) for five hours at 37°C in 10% CO₂. Supernatants were harvested and ⁵¹Cr release was detected on a TopCount counter using LumaPlate solid scintillation. Cytotoxic activity was expressed as percent specific lysis, calculated by the formula ((experimental release-spontaneous release) ÷ (maximum release-spontaneous release)) × 100.

Tissue histological examination

Transplanted and native hearts were removed and divided in half in the long axis perpendicular to the intraventricular septum. Halves were stained with hematoxylin and eosin (H&E). These were examined in a blinded fashion to determine the extent of myocardial damage, mononuclear and granulocyte cell infiltration, and vasculitis. Parallel sections were analyzed by immunohisto-chemistry on frozen tissue as previously described (8). Tissue sections were examined for immunoperoxidase staining by light microscopy for the presence of CD4 and/or CD8+ T cells.

Results

CD4 T cell In Vitro Cytotoxicity is Dependent Primarily on Fas/FasL Interaction

We first sought to determine the requirements of *in vitro* CD4 T cell killing. Cytotoxic activity of enriched, anti-BALB/c alloreactive CD4 T cells from wild-type, PFPKO, or FasL-deficient *gld* mice was assessed against MHC class II H-2^d-expressing A20 lymphoma cells *in vitro*. Purified wild-type CD8 T cells were used as positive controls as previously described (27). Results demonstrate comparable levels of lytic activity by B6 CD4 T cells, B6 CD8+ T cells, and B6 PFPKO CD4 T cells (Figure 1). However, there was scarcely detectable cytotoxic activity mediated by activated *gld* CD4 T cells. Such results are consistent with previous studies highlighting CD4 T cell-mediated cytotoxicity and the major role of Fas/FasL interactions in this process (13,14).

Perforin and Fas Represent Obligate and Parallel Pathways of CD4 T cell Mediated Cardiac Rejection

While FasL-dependent killing was a major component of CD4 T cell killing *in vitro*, sensitivity to cytotoxic effector molecules can be a function of a particular target cell. Also, it was unclear if the observed CD4 killing activity had any relevance to CD4 T cell-mediated rejection *in vivo*. To address this issue, we determined the relative requirement for T cell PFP or FasL expression and corresponding Fas expression by the cardiac allograft for rejection mediated by purified CD4 T cells. This required altering the strain combinations used to utilize B6 PFPKO and B6 *gld* cell donors and allogeneic C3H *lpr* allograft donors. Appropriate C3H or C3H*lpr* cardiac allografts were established in immune-deficient B6 *rag*^{-/-} hosts and subsequently reconstituted by the indicated source of B6 CD4 T cells (Figure 2). Without T

cell reconstitution, all allografts survived > 60 days in B6 *rag*^{-/-} hosts (data not shown). The transfer of control, wild-type B6 CD4 T cells triggered acute rejection of wild-type C3H cardiac allografts (n=4) within 17 days post-T cell reconstitution (Figure 2), consistent with previous results (7,8). Wild-type CD4 cells rejected Fas-deficient cardiac allografts with the same time frame with all allografts rejecting within 15 days. Interestingly, we found that FasL-deficient CD4 T cells triggered sluggish rejection of wild-type C3H allografts. While most allografts rejected, there was a significant delay in rejection relative to that triggered either by wild-type T cells or Fas-deficient (*lpr*) allografts. Alternatively, perforin-deficiency in B6 PFPKO CD4 T cells did not impact the tempo of acute rejection with all grafts rejecting within 13 days (Figure 2). Importantly, the *simultaneous* removal of both donor heart Fas expression (*lpr* donors) and CD4 T cell perforin expression (PFPKO T cells) completely abrogated acute rejection. That is, all C3H/*lpr* heart allografts survived >60 days post B6 *rag*^{-/-} host reconstitution with B6 PFPKO CD4 T cells ($p < 0.002$ vs. Wt C3H + B6 CD4 T cell controls, Figure 2). These results indicate that CD4 T cell-mediated cardiac rejection involves alternative but obligate contributions of allograft Fas expression and effector CD4 T cell perforin expression.

Lack of donor Fas or CD4 T cell Perforin Expression Does Not Impair T cell activation in vitro

Given the inability of PFPKO CD4 T cells to acutely reject *lpr* allografts, we set out to confirm that neither donor Fas nor T cell perforin were required for T cell activation in vitro. To address this issue, we performed mixed lymphocyte proliferation assays (MLR) with either Wt C3H or Fas-deficient C3H/*lpr* stimulator splenocytes and either naïve B6 CD4 or naïve B6 PFPKO CD4 T cells as responders (Figure 3). Results demonstrate that both Wt C3H and Fas-deficient C3H/*lpr* stimulator splenocytes induce significant proliferation of both naïve B6 CD4 T cells and B6 PFPKO CD4 T cells (Figure 3). Interestingly, there was a trend towards a greater proliferative response when stimulator splenocytes were Fas-deficient (*lpr*). Additionally, FasL-deficient B6 gld CD4 T cell anti-C3H MLRs were performed and demonstrated significant hypoproliferation consistent with the separate role of FasL in T cell activation (data not shown). Overall, these results suggest that the abrogation of acute rejection seen under circumstances of concomitant allograft Fas deficiency and CD4 T cell PFP deficiency *in vivo* is due to a defect at the level of the effector mechanism and not due to an intrinsic impairment of T cell activation by *lpr* APCs and/or PFPKO CD4 T cells.

PFPKO CD4 T cells Persist within the Allograft and Initiate Vasculopathy in Fas-Deficient LPR Allografts

As acute cardiac allograft rejection was completely abrogated *in vivo* when PFPKO CD4 T cells were transferred into hosts bearing Fas-deficient allografts, we next sought to determine the status of the surviving allografts via histologic assessment of the transplant (Figure 4). Donor cardiac allografts were harvested either at the time of clinical rejection or after 60 days of continued function. Control graft histology was prepared from control Wt C3H allografts transplanted into B6 *rag*^{-/-} recipients left unreconstituted. Such grafts had no evidence of histological rejection or vasculopathy > 60 days post-transplant (Figure 4A, panel 1). Positive control Wt C3H allografts reconstituted with naïve B6 CD4 T cells demonstrated florid lymphocytic infiltration and cardiomyocyte damage/necrosis consistent with acute cellular rejection (Figure 4A panel 2). The individual removal of either Fas from the donor heart or perforin from the CD4 T cell also resulted in graft pathology consistent with cellular rejection seen in the positive control group. Specifically, Fas-deficient C3H *lpr* allografts reconstituted with naïve B6 CD4 T cells demonstrated significant lymphocytic infiltration and cardiomyocyte damage/necrosis (Figure 4A panel 3) as did Wt C3H allografts reconstituted with naïve B6 PFPKO CD4 T cells (Figure 4A panel 4). In stark contrast, Fas-deficient C3H *lpr* cardiac allografts surviving >60 days following reconstitution with naïve B6 PFPKO CD4 T cells demonstrated moderate persistent lymphocytic infiltration without evidence of

substantial cardiomyocyte damage (Figure 4B panel 1). Immunohistochemical staining confirmed that the infiltrating cells present were CD4⁺ (Figure 4B panel 2) and CD8 and CD19 negative (data not shown). Of note, all combinations of allografts and adoptively transferred CD4 T cells demonstrated the presence of CD4⁺ cells and undetectable CD8⁺ cells as a control for the purity of the adoptively transferred cells (data not shown). Interestingly, although Fas-deficient C3H *lpr* were not actually rejected by PFPKO CD4 T cells, they did demonstrate evidence of chronic rejection/vasculopathy (Figure 4B panel 3). These occlusive vasculopathic lesions were surrounded by CD4⁺ cells, but did not demonstrate infiltration of the lesions themselves with CD4⁺ cells (Figure 4B panel 4) or CD11b⁺ macrophages (data not shown). Thus, these lesions appear to be consistent with vasculopathy and not vasculitis. Finally, C3H allografts were all histologically rejected by FasL-deficient *gld* CD4 T cells (despite 1 of 5 allografts functionally surviving >60 days) and demonstrated evidence of lymphocytic infiltration by CD4⁺ T cells and cardiomyocyte necrosis (Figure 4C panels 1–3).

Discussion

The primary requirement for CD4 T cells in acute cardiac rejection is well known (4–6,28). Traditionally, CD4 T cells are generally thought to mediate cellular destruction indirectly via CD4 T cell “help” for cytotoxic CD8 T cells (29) and B cells and/or via bystander inflammatory cytokine destruction with TNF- α or IFN- γ (30,31). However, in addition to playing a helper role in initiating rejection, CD4 T cells also can be sufficient to act as effectors of acute rejection via direct recognition of donor MHC class II molecules (7,8). Thus, while ‘indirect’ (host APC-dependent) recognition has increasingly been appreciated as a major pathway of allograft recognition, direct engagement of donor MHC antigens can be sufficient for acute rejection. While CD4 T cells can clearly reject cardiac allografts, the specific effector mechanism(s) utilized by these cells to mediate rejection was unclear. Importantly, largely in vitro studies indicate that CD4 T cells can mediate direct cellular cytotoxicity either via Fas/FasL (13,14) or via perforin-dependent (20) mechanisms. However, thus far relatively little in vivo evidence has shown a role for direct CD4 T cell toxicity in host defense (15,16,32). This study set out to determine whether Fas/FasL interactions and/or perforin played a role in the specific setting of CD4 T cell mediated cardiac allograft rejection.

Results demonstrate that CD4 T cell-mediated cardiac rejection indeed requires the alternative use of either allograft Fas expression or effector CD4 T cell perforin. It is noteworthy that while perforin appeared to play little role in CD4 T cell cytotoxicity in vitro, there was a clear requirement for perforin-dependent reactivity in vivo. Because the sensitivity to cytolytic mediators is likely dictated by the target cell used, such results highlight the importance of examining the role of candidate effector pathways in vivo. In any case, findings strongly suggest that direct cardiac allograft rejection by CD4 T cell-mediated cardiac rejection is largely dependent on cytolytic activity. Interestingly, these results are quite similar to our previous studies of CD8 T cell-mediated islet allograft rejection (27). In that report, we found that perforin and Fas also were alternative but necessary mediators of acute rejection. It is intriguing to consider that direct CD4 or CD8 T cell mediated rejection may not greatly differ in their respective mechanisms of rejection. We would propose that the mechanism of rejection (e.g. cytolytic versus other cellular or humoral pathways) may be dictated by the nature of allograft recognition (e.g. ‘direct’ versus ‘indirect’) rather than by the specific T cell subset involved. So, while CD4 T cells can certainly contribute to both acute and chronic allograft rejection in a variety of ways, when the role of the CD4 T cell is constrained to that of a primary, direct effector cell, the array of effector molecules employed may also be somewhat restricted.

A caveat to these findings is that defined immune receptors/pathways can have influences on immune reactivity beyond a defined effector role, potentially leading to ambiguous results or experimental artifacts. For example, while Fas-deficient (*lpr*) cardiac allografts were acutely

rejected by wild-type CD4 T cells, we found that there was a moderate delay in rejection by FasL (CD95L) deficient T cells. However, it is important to note that FasL clearly has been shown to play an agonist role in T cell activation and function, presumably independent of the death-inducing property of this molecule (33,34). Other studies have demonstrated a pro-costimulatory effect of Fas signaling in that Fas or FADD-deficient T cells have markedly diminished proliferative capacity (35–37). Thus, it is possible that some delay in acute rejection by *gld* T cells is due to an impaired intrinsic reactivity of these cells rather than the loss of FasL as an effector molecule. We found that *gld* CD4 T cells were almost completely non-responsive to allogeneic APCs *in vitro* (not shown), consistent with this agonist role for reverse signaling through FasL in T lymphocytes (33,34). The finding that Fas-deficient (*lpr*) allografts do *not* have prolonged survival would be consistent with this interpretation of the results. Thus, experimental results must be viewed considering the potential multiple properties of immunoreceptors/effector molecules in immune responses.

The histologic evaluation of cardiac allografts in this study mirrored our *in vivo* adoptive transfer experimental findings. More specifically, whereby individual deficiency in either donor Fas or CD4 T cell perforin demonstrated acute cellular rejection, the simultaneous removal of donor Fas and CD4 T cell perforin only demonstrated infiltration by CD4 T cells without evidence of significant cardiomyocyte damage. This histologic appearance strongly suggests that activated CD4 T cells are able to home to the graft (intact Signal 1, Signal 2 and MHC-restricted targeting), but once there are incapable of acutely rejecting the graft (loss of effector CD4 T cell function). It is interesting to note that while Fas-deficient allografts are not acutely rejected by PFPKO CD4 T cells, they do develop evidence of vasculopathy (chronic rejection). As MHC Class II expression is intact on both the donor allograft and the host tissues, both the direct and indirect pathways of antigen presentation are operational. As previous work in our laboratory has shown that MHC Class II deficient (B6 C2D) donor allografts are not acutely rejected by naïve CD4 T cells in immune deficient CB.17 SCID hosts but do develop evidence of vasculopathy (7), the current results again implicate an *indirect* CD4 T cell in the pathogenesis of chronic rejection (vasculopathy). This result is not completely surprising as other studies have also implicated the indirect CD4 T cell in the pathogenesis of chronic cardiac allograft rejection (38,39). Importantly, this finding demonstrates the utility of this model for the future study of the molecular pathogenesis of vasculopathy initiated by indirect CD4 T cells.

In conclusion, results of these studies demonstrate that acute CD4 T cell-mediated cardiac allograft rejection requires donor Fas and CD4 T cell perforin expression in an obligate and parallel fashion. Additionally, the simultaneous loss of donor Fas expression and effector CD4 T cell perforin expression abrogates rejection by impairing the effector mechanism of the CD4 T cell and not by inhibiting recognition (Signal 1) or costimulation (Signal 2). To our knowledge, this is the first demonstration of a requirement for a directly cytotoxic CD4 T cell in an *in vivo* model of transplantation. Consequently, potential therapies to abrogate clinical acute cardiac rejection should take into consideration the potential need for inhibiting the required molecular machinery for direct CD4 CTL function. Additionally, these results highlight the redundancy of the immune system and underscore the probability that therapies will likely require more than the inhibition or targeting of individual molecules or pathways to be effective. Finally, the loss of donor Fas expression and CD4 T cell perforin expression completely abrogated acute rejection but did not impair the development of chronic rejection (vasculopathy). This finding implicates the indirect CD4 T cell in the pathogenesis of vasculopathy and demonstrates that the development of vasculopathy initiated by CD4 T cells occurs independently of allograft Fas and CD4 T cell perforin expression.

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Abbreviations

PFP	<i>Perforin</i>
PFPKO	<i>Perforin Knockout</i>
C2D	<i>MHC Class II Deficient</i>
<i>lpr</i>	<i>Lymphoproliferative (Fas-deficiency)</i>
<i>gld</i>	<i>Generalized lymphoproliferative disease (FasL-deficiency)</i>

References

1. Valentine H. Cardiac allograft vasculopathy after heart transplantation: risk factors and management. *J Heart Lung Transplant* 2004;23 (5 Suppl):S187. [PubMed: 15093804]
2. Bennett LE, Keck BM, Hertz MI, Trulock EP, Taylor DO. Worldwide thoracic organ transplantation: a report from the UNOS/ISHLT international registry for thoracic organ transplantation. *Clin Transpl* 2001;25. [PubMed: 12211789]
3. Hunt S, Billingham M. Long-term results of cardiac transplantation. *Annu Rev Med* 1991;42:437. [PubMed: 1827971]
4. Yin D, Fathman CG. CD4-positive suppressor cells block allotransplant rejection. *J Immunol* 1995;154 (12):6339. [PubMed: 7759872]
5. Herbert J, Roser B. Strategies of monoclonal antibody therapy that induce permanent tolerance of organ transplants. *Transplantation* 1988;46 (2 Suppl):128S. [PubMed: 3136565]
6. Shizuru JA, Seydel KB, Flavin TF, et al. Induction of donor-specific unresponsiveness to cardiac allografts in rats by pretransplant anti-CD4 monoclonal antibody therapy. *Transplantation* 1990;50 (3):366. [PubMed: 1976282]
7. Pietra BA, Wiseman A, Bolwerk A, Rizeq M, Gill RG. CD4 T cell-mediated cardiac allograft rejection requires donor but not host MHC class II. *J Clin Invest* 2000;106 (8):1003. [PubMed: 11032860]
8. Grazia TJ, Pietra BA, Johnson ZA, Kelly BP, Plenter RJ, Gill RG. A two-step model of acute CD4 T cell mediated cardiac allograft rejection. *J Immunol* 2004;172 (12):7451. [PubMed: 15187123]
9. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H. Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annu Rev Immunol* 1996;14:207. [PubMed: 8717513]
10. Siegel RM, Chan FK, Chun HJ, Lenardo MJ. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 2000;1 (6):469. [PubMed: 11101867]
11. Hanabuchi S, Koyanagi M, Kawasaki A, et al. Fas and its ligand in a general mechanism of T cell-mediated cytotoxicity. *Proc Natl Acad Sci U S A* 1994;91 (11):4930. [PubMed: 7515183]
12. Kagi D, Vignaux F, Ledermann B, et al. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* 1994;265 (5171):528. [PubMed: 7518614]
13. Ju ST, Cui H, Panka DJ, Ettinger R, Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by CD4+ Th1 and CD8+ cytotoxic T cells. *Proc Natl Acad Sci U S A* 1994;91 (10):4185. [PubMed: 7514297]
14. Stalder T, Hahn S, Erb P. Fas antigen is the major target molecule for CD4+ T cell-mediated cytotoxicity. *J Immunol* 1994;152 (3):1127. [PubMed: 7507960]
15. Seino K, Kayagaki N, Bashuda H, Okumura K, Yagita H. Contribution of Fas ligand to cardiac allograft rejection. *Int Immunol* 1996;8 (9):1347. [PubMed: 8921411]
16. Larsen CP, Alexander DZ, Hendrix R, Ritchie SC, Pearson TC. Fas-mediated cytotoxicity. An immunoeffector or immunoregulatory pathway in T cell-mediated immune responses? *Transplantation* 1995;60 (3):221. [PubMed: 7544034]

17. Strehlau J, Pavlakakis M, Lipman M, et al. Quantitative detection of immune activation transcripts as a diagnostic toll in kidney transplantation. *Proc Natl Acad Sci U S A* 1997;97:695. [PubMed: 9012847]
18. Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 2001;344:947. [PubMed: 11274620]
19. Simon T, Opelz G, Wiesel M, Ott RC, Susal C. Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. *Am J Transplant* 2003;3:1121. [PubMed: 12919092]
20. Hombach A, Heuser C, Marquardt T, et al. CD4+ T cells engrafted with a recombinant immunoreceptor efficiently lyse target cells in a MHC antigen- and Fas-independent fashion. *J Immunol* 2001;167 (2):1090. [PubMed: 11441120]
21. Adhikary D, Behrends U, Moosmann A, Witter K, Bornkamm GW, Mautner J. Control of Epstein-Barr virus infection in vitro by T helper cells specific for virion glycoproteins. *J Exp Med* 2006;203 (4):995. [PubMed: 16549597]
22. Jellison ER, Kim SK, Welsh RM. Cutting edge: MHC class II-restricted killing in vivo during viral infection. *J Immunol* 2005;174 (2):614. [PubMed: 15634878]
23. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 1992;356 (6367): 314. [PubMed: 1372394]
24. 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. [PubMed: 4583148]
25. Sarmiento M, Glasebrook AL, Fitch FW. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytotoxicity in the absence of complement. *J Immunol* 1980;125 (6):2665. [PubMed: 6159417]
26. Glimcher LH, Kim KJ, Green I, Paul WE. Ia antigen-bearing B cell tumor lines can present protein antigen and alloantigen in a major histocompatibility complex-restricted fashion to antigen-reactive T cells. *J Exp Med* 1982;155 (2):445. [PubMed: 6460073]
27. Sleater M, Diamond AS, Gill RG. Islet allograft rejection by contact-dependent CD8+ T cells: perforin and FasL play alternate but obligatory roles. *Am J Transplant* 2007;7 (8):1927. [PubMed: 17617855]
28. Pietra B, Rizeq M, Bolwerk A, Gill RG. CD4 T cells acutely reject cardiac allografts via direct donor recognition. *Transplant Proc* 1999;31 (1-2):92. [PubMed: 10083022]
29. Buller RM, Holmes KL, Hugin A, Frederickson TN, Morse HC 3rd. Induction of cytotoxic T cell responses in vivo in the absence of CD4 helper cells. *Nature* 1987;328 (6125):77. [PubMed: 2955227]
30. Bishop DK, Chan S, Li W, Ensley RD, Xu S, Eichwald EJ. CD4-positive helper T lymphocytes mediate mouse cardiac allograft rejection independent of donor alloantigen specific cytotoxic T lymphocytes. *Transplantation* 1993;56 (4):892. [PubMed: 8212213]
31. Nickerson P, Steiger J, Zheng XX, et al. Manipulation of cytokine networks in transplantation: false hope or realistic opportunity for tolerance? *Transplantation* 1997;63 (4):489. [PubMed: 9047139]
32. Schulz M, Schuurman HJ, Joergensen J, et al. Acute rejection of vascular heart allografts by perforin-deficient mice. *Eur J Immunol* 1995;25 (2):474. [PubMed: 7533086]
33. Suzuki I, Fink PJ. Maximal proliferation of cytotoxic T lymphocytes requires reverse signaling through Fas ligand. *J Exp Med* 1998;187:123. [PubMed: 9419218]
34. Suzuki I, Fink PJ. The dual functions of Fas ligand in the regulation of peripheral CD8+ and CD4+ T cells. *Proc Natl Acad Sci U S A* 2000;97 (4):1707. [PubMed: 10677522]
35. Kennedy NJ, Kataoka T, Tschopp J, Budd RC. Caspase activation is required for T cell proliferation. *J Exp Med* 1999;190 (12):1891. [PubMed: 10601363]
36. Alam A, Cohen LY, Aouad S, Sekaly RP. Early activation of caspases during T lymphocyte stimulation results in selective substrate cleavage in nonapoptotic cells. *J Exp Med* 1999;190 (12): 1879. [PubMed: 10601362]
37. Zhang J, Cado D, Chen A, Kabra NH, Winoto A. Fas-mediated apoptosis and activation-induced T cell proliferation are defective in mice lacking FADD/Mort1. *Nature* 1998;392 (6673):296. [PubMed: 9521326]

38. Yamada A, Laufer TM, Gerth AJ, et al. Further analysis of the T cell subsets and pathways of murine cardiac allograft rejection. *Am J Transplant* 2003;3 (1):23. [PubMed: 12492706]
39. Szeto WY, Krasinskas AM, Kreisel D, Krupnick AS, Popma SH, Rosengard BR. Depletion of recipient CD4+ but not CD8+ T lymphocytes prevents the development of cardiac allograft vasculopathy. *Transplantation* 2002;73 (7):1116. [PubMed: 11965043]

Figure 1. CD4 T cell Cytotoxicity is Mediated Primarily by Fas/FasL interaction

Cytotoxic T-lymphocyte Assays (CTL) demonstrate that activated B6 CD4 T cells, B6 PFPKO CD4 T cells, and B6 CD8 T cells are capable of directly killing MHC Class I and II bearing BALB/c-derived A20 target cells. However, activated Fas Ligand-deficient *gld* CD4 T cells demonstrate very little lysis of A20 target cells. Data are representative of three experiments testing CD4 and CD8 T cell direct cytotoxicity against BALB/c-derived A20 B-cell lymphoma cells.

Figure 2. Perforin and Fas Represent Obligate and Parallel Pathways of CD4 T cell-Mediated Cardiac Rejection

B6, B6 PFPKO, and B6 *gld* CD4 T cells were utilized to reconstitute B6 *rag*^{-/-} recipients of C3H or Fas-deficient C3H/*lpr* cardiac allografts. Removal of Fas alone (◆, *p*=NS vs. control Wt C3H + B6 CD4 T cells) from the donor hearts or removal of perforin alone from the CD4 T cells (■, *p*=NS vs. control Wt C3H + B6 CD4 T cells) did not abrogate rejection. Interestingly, the removal of FasL from effector CD4 T cells did delay rejection significantly (●, *p* < 0.02 vs. Wt C3H + B6 CD4 T cells, *p* < 0.01 vs. Wt C3H + B6 PFPKO CD4 T cells, and *p* < 0.01 vs. C3H/*lpr* + B6 CD4 T cells). However, most allografts were still rejected (4 of 5).

Significantly, the simultaneous removal of both donor Fas and CD4 T cell perforin completely abrogated rejection (○, *p* < 0.002 vs. control Wt C3H + B6 CD4T cells, Wt C3H + B6 PFPKO CD4 T cells, and C3H/*lpr* + B6 CD4 T cells). This abrogation was significantly more robust than the individual removal of CD4 T cell FasL (○, *p* < 0.003 vs. Wt C3H + B6 *gld* CD4 T cells).

Figure 3. B6 and B6 PFPKO CD4 T cells Proliferate to C3H Wt and Fas-deficient LPR Stimulators
Mixed lymphocyte assays demonstrate that Fas-deficient C3H LPR stimulator cells elicit vigorous proliferative responses from both Wt B6 and Perforin-deficient B6 PFPKO CD4 T cells. Data are representative of three experiments testing CD4 T cell-dependent reactivity to donor splenocytes. Error bars represent the standard deviation from quadruplicate wells in the MLR assay.

Figure 4. Histologic Assessment of Heart Allografts

A. (Panel 1): Wild type C3H allograft un-reconstituted at >60 days demonstrates no significant cellular infiltration and intact cardiomyocytes. (Panel 2): Wild type C3H allograft reconstituted with B6 CD4 T cells rejected at 10 days post reconstitution, demonstrates significant cellular lymphocytic infiltration and cardiomyocyte damage/necrosis. (Panel 3): Fas-deficient C3H *lpr* allograft reconstituted with B6 CD4 T cells rejected at 9 days post reconstitution, demonstrates significant cellular lymphocytic infiltration and cardiomyocyte damage/necrosis. (Panel 4): Wild type C3H allograft reconstituted with B6 PFPKO CD4 T cells rejected at 9 days post reconstitution, demonstrates significant cellular lymphocytic infiltration and cardiomyocyte damage/necrosis.

B. (Panel 1): Fas-deficient C3H *lpr* allograft reconstituted with B6 PFPKO CD4 T cells at >60 days post reconstitution, demonstrates moderate cellular lymphocytic infiltration and no significant cardiomyocyte damage. (Panel 2): Immunoperoxidase staining for CD4 shows CD4 + infiltrating cells in a Fas-deficient C3H *lpr* allograft reconstituted with B6 PFPKO CD4 T cells surviving >60 days. (Panel 3): Fas-deficient C3H *lpr* allograft reconstituted with B6 PFPKO CD4 T cells at >60 days post reconstitution demonstrates a vasculopathic lesion. (Panel 4): Immunoperoxidase staining for CD4 of the same vasculopathic lesion demonstrates CD4 + cells surrounding the lesion without infiltration of the lesion itself.

C. (Panel 1): Wt C3H allograft reconstituted with B6 *gld* CD4 T cells rejected at 42 days post reconstitution, demonstrates significant severe cellular lymphocytic infiltration and cardiomyocyte damage/necrosis. (Panel 2): Wt C3H allograft reconstituted with B6 *gld* CD4 T cells at >60 days post reconstitution, demonstrates moderate to severe cellular lymphocytic infiltration and cardiomyocyte damage/necrosis. (Panel 3): Immunoperoxidase staining for CD4 shows CD4+ infiltrating cells in a Wt C3H allograft reconstituted with B6 *gld* CD4 T cells surviving >60 days.