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OVERCOMING CARDIAC ALLOGRAFT VASCULOPATHY (CAV) BY INDUCING TOLERANCE

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1. ABSTRACT

There is compelling evidence that MHC-driven immune processes play a dominant role in the development of cardiac allograft vasculopathy. Thus, it makes intuitive sense that tolerance, which eliminates donor alloreactivity, should protect against CAV. However, in the experimental literature, there are examples of CAV occurring in recipients rendered tolerant by either peripheral or central induction protocols. Why does transplant arteriopathy occur in recipients that have achieved a robust state of tolerance or in the animals devoid of T or B cell immunity? There may be immunological blindspots that persist even after a state of tolerance is achieved. These blindspots could contribute to the pathogenesis of chronic rejection (CR).

2. INTRODUCTION

A major remaining barrier to the success of solid organ transplantation is chronic rejection. Despite advances in surgical technique, development of new immunosuppressive agents, and advances in therapeutic protocols, the majority of solid organ allografts eventually show a progressive decline in function leading ultimately to graft failure. Recent evidence suggests that long-term graft survival times may be improving for some organs (1); however, the relationship of these data to the incidence of CR remains unclear. A significant body of evidence exists implicating MHC-driven immune processes as the primary cause of chronic rejection. Thus, it makes sense that the induction of immunological tolerance, which eliminates donor alloreactivity, should protect against chronic

rejection. Given the known complications associated with current immunosuppressive regimens, such as susceptibility to infection, organ specific drug toxicity, and transplant-associated malignancies, increasing attention has focused on the possibility of inducing tolerance to a transplanted organ. Such a strategy, if successful, would have significant medical and lifestyle implications for patients with end-stage organ failure. However, there are examples in the literature of chronic rejection occurring in the face of apparently successful tolerance induction (as defined by long-term graft survival, acceptance of a donor-specific challenge graft, rejection of a third-party challenge graft). In light of these counterintuitive findings, this review was undertaken to examine the effect of tolerance induction on the development of chronic rejection. First, the pathologic features and mechanisms of chronic rejection are reviewed. Second, the effects of central and peripheral tolerance are chronic rejection in experimental models of transplantation are examined. Then, possible explanations for the presence of chronic rejection lesions in apparently tolerant organ recipients are discussed.

2.1. Pathology of chronic rejection

Chronic rejection leads to a gradual, progressive deterioration in graft function months to years after transplantation. While the clinical signs of chronic rejection (CR) are organ specific, the underlying pathology in every organ system studied is characterized by a progressive narrowing of luminal graft structures. In the kidney, this process, termed chronic allograft nephropathy (CAN), consists of obliterative vasculopathy, interstitial fibrosis with tubular atrophy, and glomerulosclerosis (2,3). In the heart, intimal proliferation and subsequent luminal occlusion of the coronary vasculature is referred to as cardiac allograft vasculopathy (CAV). The lesions of CAV bear some morphological similarity to coronary lesions occurring in non-transplanted patients; however, the hallmark of CAV in a transplanted heart is the diffuse and concentric nature of the lesions, which usually progress inexorably to graft loss. The end-point of CAV is congestive heart failure, arrhythmias, myocardial infarction, and sudden death. While vascular lesions also occur in the lung, the characteristic pulmonary manifestation of chronic rejection is obliterative bronchiolitis (OB), which is characterized by the progressive narrowing of smaller airways, leading eventually to loss of air exchange capacity of the transplanted organ. In the liver, the term “vanishing bile duct syndrome” has been applied to a process similar in histology to primary biliary cirrhosis. Although lower in frequency than that seen in other organs, these lesions share features in common with other forms of chronic rejection, and often appear in association with vascular lesions (4).

2.2. Demographics of chronic rejection

The incidence of CAV is approximately 10% per year. Obliterative bronchiolitis occurs at a rate of greater than 15% per year, with a prevalence of 60-80% at 5 years (5). In the kidney, the histopathologic changes of chronic allograft nephropathy occur with an incidence of 62-72% in protocol biopsies of cadaveric renal allografts taken at two years (6). There is evidence that long-term survival of renal allografts may be improving (1), however, it is not yet clear whether this trend represents a decrease in CR. While newer immune suppressive regimens have reduced the incidence of *acute* rejection in heart and lung transplantation, no improvement in long-term outcomes for thoracic transplantation has been observed. Thus, CR remains a significant problem in solid organ transplantation, and remains the most common cause of late graft loss (7).

3. MECHANISMS OF CHRONIC REJECTION

Potential mechanisms of chronic rejection have generally been categorized as alloantigen-dependent or alloantigen-independent (reviewed in (7)). The first category represents those phenomena driven by MHC disparities between donor and host, resulting in early acute immunologic injury and a subsequent ongoing host anti-donor alloresponse. The second category has been thought to consist of donor-associated factors relating to age and disease state of the organ, the clinical condition of the donor, and factors related to the organ harvest, implantation, and subsequent therapy. While this represents a convenient categorization, it is increasingly clear that there are significant interactions between immune and non-immune stimuli in the pathogenesis of chronic rejection. Therefore, in reviewing the factors contributing to chronic rejection, we will divide them into mechanisms that are directly MHC-dependent and those that affect or are affected by the MHC in an indirect manner.

3.1 Mechanisms directly associated with the MHC

3.1.1. MHC disparities

Both animal studies and clinical data support the idea that disparities in alloantigen between donor and recipient correlate with strength of rejection, and there is ample evidence to suggest that long term graft survival is proportionate to the degree of MHC matching (Reviewed in (3)). In human renal allografts, every HLA mismatch decreases long-term graft survival by 5% (8). In protocol renal biopsies of patients without evidence of acute rejection, pathologic changes of CR were evident in HLA mismatched, but not HLA-identical grafts (9).

3.1.2. Acute Rejection

Early episodes of acute rejection correlate strongly with graft survival (8). There is an increased incidence of CR in patients having more than one episode of acute rejection (10). There is also a correlation between severity of acute rejection episodes and CR (11). Intimately associated with incidence of acute rejection is level of immune suppression. Almond *et al*. (2) identified a cyclosporine dosage of < 5 mg/kg/day at one year as a significant risk factor in the development of chronic rejection. As is discussed below, calcineurin inhibitors have their own inherent toxicity, which may also contribute to chronic rejection. As newer agents become available and regimens are altered to include them, the definition of “appropriate” levels of immune suppression will have to be reassessed. This will be especially true when evolving tolerance protocols are applied to the clinic, where classical immune suppression could be detrimental.

3.1.3. Indirect allorecognition and epitope spreading

One theory explaining the pathogenesis of chronic rejection suggests that following transplantation, a small number of T cells are indirectly primed against a restricted repertoire of immunodominant peptides (12). There are two distinct, yet non-mutually exclusive, pathways of T cell allorecognition. Direct allorecognition occurs when host T cells recognize intact allo-MHC molecules on the surface of donor cells (usually APCs, such as dendritic cells or macrophages). Indirect recognition occurs when host T cells respond to processed alloantigen presented as peptides by self-APCs (13-16). During this process, alloantigen is shed from the donor graft, taken up, processed, and presented to host T cells by host APCs, analogous to self-restricted T cell recognition of nominal antigen (reviewed in (17-19)). Early posttransplant, the actions of these self-MHC restricted T cells are overshadowed by the larger number of T lymphocytes that are directly primed by professional APCs present in the newly engrafted tissue (i.e. donor passenger leukocytes) (20). However, as passenger leukocytes are depleted from the allograft over time (21) and direct allorecognition diminishes in importance (22,23), the persistent, albeit low-grade alloresponse perpetuated by the indirectly primed T cells, predominates. These cells would then mediate chronic rejection by either providing help for alloantibody formation and/or by promoting lymphokine secretion required for macrophage and cytotoxic T cell activity (12,24,25).

The importance of indirect pathway in the pathogenesis of CAV in large animals was recently demonstrated by Lee *et al* (26) who showed that miniature swine immunized with a panel of donor-MHC derived peptides prior to transplantation developed an accelerated and severe form of CAV compared to unprimed controls (26). Recent studies have demonstrated the persistence of donor specific MHC allopeptide T cell reactivity in humans with chronic rejection of cardiac (24,27), kidney (28), and lung (29) allografts. These longitudinal studies have also shown that self-MHC restricted T helper cells are able to recognize new allodeterminants (donor peptides) and thus shift or expand the host's T cell repertoire over time through a process termed epitope spreading (24,28,30).

Taken together, these data suggest a means by which alloreactivity may emerge despite an initial success in inducing tolerance. If initially quiescent allodeterminants expressed on the donor organ gain immunogenicity over time through the process of epitope spreading, early tolerance induction protocols may be unable to prevent the host's T cells from responding to them later. Thus, a truly complete and effective tolerance induction strategy must be designed not only to prevent T cell activation occurring through both the direct and indirect pathways of allorecognition, but must be able to actively suppress alloreactivity that develops late through the process of epitope spreading.

3.2. Mechanisms indirectly associated with the MHC

3.2.1. Ischemia/reperfusion

The study of ischemia-reperfusion injury has implications for every aspect of organ transplantation. The importance of careful organ harvest, preservation, and transport protocols in initial organ function have long been clear (31). Despite longstanding interest in this subject, the effects of ischemia-reperfusion injury on CR are only beginning to be understood (32). Initial ischemia and subsequent reperfusion, trigger release of reactive oxygen species and other acute inflammatory mediators (33). Activated endothelium expresses adhesion molecules, leading to extravasation of polymorphonuclear leukocytes and continuation of the inflammatory stimulus. Upregulation of MHC molecules on activated endothelium has also been demonstrated in experimental models of cold ischemia (34). This suggests that ischemia-reperfusion injury may lead to a generalized increase in immunogenicity of the graft, which could affect subsequent T-cell activity in the host. Whether ischemia-reperfusion injury necessarily leads to CR in the absence of an alloimmune response is not clear, however. Some evidence suggests that established tolerance might be protective, even in the setting of prolonged ischemia (35).

3.2.2. Infections

The effects of cytomegalovirus (CMV) mediated injury on the pathogenesis of CR have been observed in both the clinical and the experimental realm. Several studies have implicated CMV-mediated injury to endothelium as a contributor to allograft atherosclerosis (11). CMV infection may act in a number of ways to contribute to this process, for example, through upregulation of vascular adhesion molecules (36) as a consequence of direct activation of immune cells, or through viral peptide sequence homology to human HLA antigens (molecular mimicry) (37). Of note, clinical data from renal transplant patients indicate that CMV infection may contribute to the pathogenesis of CR only if there is evidence of acute rejection (11), which is additional evidence of the interaction between "non-immune" and MHC-driven events in CR.

3.2.3. Donor factors/senescence

Given the pervasive shortage of donor organs and increasing demand for the existing organ pool, attempts are underway to increase the numbers of available organs through the use of older, hypertensive or diabetic donors (38). Organs from such donors may have a number of characteristics, which make them more susceptible to CR. Underlying organ injury from whatever source may lead to subclinical T-cell activation, which is not easily measured with currently available assays. In clinical renal transplantation, low nephron mass in kidneys transplanted from older donors is thought to contribute to more rapid decline in graft function (39), a finding supported by rodent experiments (40).

That both immune and non-immune pathways contribute to graft injury in organ transplantation is not disputed. Halloran *et al.* (41) have proposed that the combined injury resulting from transplantation and subsequent immune-mediated injury may exhaust the endogenous repair capacity of transplanted tissues. The pathologic hallmarks of ageing in tissues include slower repair after injury, arterial intimal thickening, calcification,

and interstitial fibrosis. In the naïve host, these processes advance slowly over a lifetime, and do not normally limit organ function significantly. The cellular senescence theory suggests that these processes result from a loss of the ability of key cell populations to proliferate in response to injury. After transplantation, these cell populations might reach senescence more rapidly, leading to atrophy and fibrosis in vascular endothelium, bronchial and tubular epithelium. As the injury leading to accelerated senescence results from a combination of factors, including MHC-driven immune mediated injury, successful tolerance might be expected to slow this process, ideally to something approaching the native state.

3.2.4. Brain death

Brain death and the subsequent systemic physiologic derangements in the donor are thought to be related to early and late organ dysfunction (42). Changes across multiple organ systems have been defined in brain dead donors. This may result from a general upregulation of the inflammatory response, with activation of endothelium and the innate immune system (see below) as well as hemodynamic instability in the early period following brain death, leading to end-organ ischemia. Of note, acute rejection episodes are both more common and more severe in cadaveric compared with living-related organ transplantation (43).

3.2.5. Drug toxicity

Transplant patients who have experienced episodes of FK506 or cyclosporine toxicity have been found to have a higher incidence of CAN at protocol biopsies than patients whose drug levels have been better controlled. To date, the effect of CsA and FK506 toxicity on the development of CR is best demonstrated in the kidney, which may indicate a longer-term effect of the known nephrotoxicity of these drugs, rather than direct exacerbation of CR *per se*. Steroids may contribute to CR in a number of ways. In a variety of experimental models, steroids have been found to interfere with the induction of tolerance (44,45). In both small (45) and large (46) animal studies of tolerance induction via costimulatory blockade, administration of steroids in the early post-transplant period abrogated tolerance induction. The ongoing dominance of corticosteroids in current era anti-rejection therapy may make it difficult to bring successful tolerance protocols to the clinic.

3.2.6. Hypercholesterolemia

Similarities between the pathology of native vessel arteriosclerosis and chronic rejection have led many to postulate an interaction between immune causes and hypercholesterolemia in the genesis of these lesions. Hyperlipidemia is common after organ transplantation, affecting up to 80% of heart transplant recipients (47). Cholesterol lowering drugs are now widely used in transplant recipients. Two randomized, controlled trials in heart transplant recipients have demonstrated that HMG-CoA reductase inhibitors, the so-called "statin" drugs, improve cardiac allograft survival and decrease the rate of coronary disease in the donor organ (48,49). More recent evidence has pointed to a dual role for lipid lowering drugs in reducing the stimulus for vascular lesions to develop. In vitro studies have demonstrated inhibition of NK cell activity by these drugs (50), suggesting that reduction in activity of the innate immune system may also contribute to a reduced risk of atherosclerosis.

3.2.7. Innate immunity and cytokine excess

While much basic research in transplantation focuses on T-cell responses to alloantigen, the role of macrophages in allograft rejection has received increasing attention. A number of stimuli associated with transplantation activate macrophages, including the inflammatory state associated with ischemia-reperfusion, release of cytokines from activated immune cells, and tissue injury associated with surgery. Activated macrophages secrete cytokines, promote antigen recognition, chemotaxis, adhesion molecule expression, and are intimately involved in endothelial and smooth muscle activation and proliferation (51). Interferon-γ, produced by activated T and NK cells, has wide-ranging effects on endothelium and deserves special mention. In the mouse, treatment with interferon-γ monoclonal antibody inhibits the development of CAV (52) and hearts transplanted into interferon-γ knockout mice also develop fewer CAV lesions (53). Even more striking is a recent study by Tellides and colleagues (54) who found that interferon-γ elicited intimal proliferation in transplanted vessels without need for leukocytes. Taken together, these studies and others suggest a central role for interferon-γ in the pathogenesis of CR.

NK cells have been implicated in CR both as cytokine producers (interferon-γ) and in NK cell mediated cytotoxicity to transplanted cells. Blockade of NK cell activity in $CD28^{-/-}$ knockout mice, which normally reject cardiac allografts, led to prolonged graft survival (55). Russell and colleagues demonstrated CAV lesions in T and B cell deficient mice ($RAG/1^{-/-}$) after heterotopic heart transplantation (56). The authors explored the potential role for NK cells in generating these lesions by transplanting hearts into SCID/beige mice, which have impaired NK cell cytolytic activity. These animals also developed CAV lesions, although of lesser severity (56). One explanation for these findings is that NK cell production of interferon-γ, rather than direct cytotoxicity to transplanted tissues, was responsible for the generating vascular lesions in the transplanted hearts.

3.2.8. Transplantation-induced autoimmunity

Fedoseyeva *et al*. (57) have found evidence for *de novo* autoimmunity to cardiac myosin, an autologous contractile protein specific for cardiac tissue, in both mouse and human recipients of cardiac allografts. In mice, organ-specific autoimmunity persisted in animals that developed chronic rejection (Fedoseyeva E.V., manuscript submitted). These findings present the intriguing possibility that autoantigens specific to transplanted tissues may be "uncovered" due to tissue injury during or after transplantation, and then serve as a stimulus for ongoing autoimmune responses, even in the face of tolerance to donor-specific antigens. Organ nonspecific autoimmunity has also been described in a rat cardiac model, in which

autoreactive T-cells specific for heat shock protein derived peptides were isolated from chronically rejecting cardiac allografts (58).

4. TOLERANCE AND CARDIAC ALLOGRAFT VASCULOPATHY

There is compelling evidence that MHC-driven immune processes play a dominant role in the development of cardiac allograft vasculopathy (59). Thus, it makes intuitive sense that tolerance, which eliminates donor alloreactivity, should protect against CAV. However, in the experimental literature there are examples of CAV occurring in recipients rendered tolerant by either peripheral or central induction protocols. For instance, despite fulfilling generally accepted criteria for peripheral tolerance (i.e. long-term graft survival, acceptance of a donor-specific challenge graft, and rejection of a third-party challenge graft), cardiac allografts in several systems developed arteriopathy (60,61). Our own results have demonstrated that the induction of central tolerance through the establishment of multilineage mixed chimerism diminishes but does not preclude the development of CAV in either small (56) or large animals (62,63). Furthermore, allografts transplanted into $RAG1^{-/-}$ recipients, which are profoundly incapable of adaptive immune responses, still developed proliferative coronary vascular lesions, while isotransplants between members of these deficient strains were generally free of coronary vascular lesions (64). Why does transplant arteriopathy occur in recipients that have achieved a robust state of tolerance or in the animals devoid of T or B cell immunity? Potential reasons will be discussed below, after detailing the mechanisms of tolerance and some of the relevant experimental studies.

4.1 Mechanisms of tolerance

Transplantation tolerance can be defined as loss of reactivity to the histocompatibility antigens expressed by the donor graft, with maintenance of full reactivity to all other non-self antigens. Tolerance induced in the thymus is often referred to as “central tolerance”, in distinction from "peripheral tolerance", which occurs outside the thymus.

4.1.2. Central tolerance

Central tolerance is thought to be a process analogous to self tolerance, in which T-cell clones with a high affinity for self antigens are deleted at an early stage of development (clonal deletion). If alloantigens are properly presented to developing T-cells in the recipient thymus, donor-specific clonal deletion likely takes place, resulting in a T-cell repertoire that is unresponsive to donor antigens as well as self. The capacity of bone marrow transplants to induce tolerance has been recognized for many years, (65-68) and results in large part from the ability of bone-marrow derived cells to migrate to the thymus. Several bone-marrow derived cell types, including dendritic cells (69,70) and B cells (70), have been shown to have the capacity to induce such thymic deletion. In addition, central tolerance might be achieved via direct injection of cells or antigen into the thymus, (71,72,73) or via transplantation of vascularized donor thymic tissue. (74,75). However, deletion is probably not the only mechanism by which tolerance is induced intrathymically, as there is some evidence that both anergy (76) and suppression (77) can occur in the thymus.

4.1.2. Peripheral tolerance

Peripheral tolerance is induced when T-cell clones that escape negative selection in the thymus are made anergic (clonal anergy) or become subject to regulatory mechanisms (specific suppression/regulation) in the periphery. Such a process is postulated to be responsible for tolerance to innocuous environmental antigens encountered via respiratory or enteral exposure, and to newly uncovered self antigens as part of normal tissue turnover. T cells require two distinct signals for full activation. The first signal is provided by the engagement of the TCR with the MHC plus peptide complex on APCs, and the second "costimulatory" signal is provided by engagement of one or more T cell surface receptors with their ligands on APCs. Signaling through the TCR alone without a costimulatory signal leads to a prolonged state of T cell anergy (reviewed in (78,79)). One major costimulatory signal is that provided by interaction of CD28 on T cells with either of its two ligands, B7-1 or B7-2 on APCs. Also of great interest is the role of CD40 and its ligand as they relate to the process of allograft rejection (reviewed in (78)). The role of CD40L in direct T cell activation is uncertain (80). It is not known whether CD40L acts directly to transduce a costimulatory signal to the T cell, or indirectly to induce CD28-ligands, or other costimulatory molecules on APCs (81,82). However, antibodies to CD40L are extremely effective in preventing acute graft rejection in a mouse model of vascularized cardiac allografts (83-86).

While the existence of regulatory T cells has been demonstrated in diverse models of transplant tolerance, the phenotype and specificity of these cells in vivo remains elusive (87). Rodent studies have focused on the CD4+, CD25+ T-cell population as the cell type involved in maintaining tolerance (88). Although controversy still surrounds the exact role and function of these cells, CD4+CD25+ cells have consistently been shown to suppress the alloresponses of $CD4^{+}CD25^{-}$ cells as well as $CD8^{+}$ responders (89). Once activated, these cells suppress T cell activation in an antigen-independent manner (90,91). They appear to require activation through the TCR to induce suppression (92), act through an APC-independent mechanism (93), are cytokine-independent, and are generated primarily in the thymus, with CD25 appearing during the transition of $CD4^{+}CD8^{+}$ double positive cells to single positive $CD4^{+}$ cells (91,94). While initial in vivo experiments implicated CD4+CD25+ T-cells as regulators of autoimmunity, more recent studies in murine transplant systems support a role for these cells in tolerance induction (95-97). Adoptive transfer of these cells leads to infectious tolerance, and spontaneous acceptance of renal allografts in mice is associated with donor-reactive, cell mediated immune regulation (98). In addition, Taylor *et al*. (99), have demonstrated that CD4+CD25+ regulatory T cells are required for costimulatory blockade-induced allotolerance in a murine *ex vivo* tolerance induction model. This cell population has been found to exist in humans, with a

similar frequency (5-15%) to that seen in rodents, and in vitro studies have confirmed a regulatory function for human CD4+CD25+ cells (91,100).

4.2. Assessment of tolerance

There are several difficulties in interpreting the experimental literature on the effects of tolerance on chronic rejection. One problem is that the endpoints used to assess outcomes in most animal studies of transplantation are of short duration compared with the endpoints used to assess outcomes in human transplantation. For instance, in rodent models, graft survival to an arbitrary 100 days has historically been considered a valid endpoint for the successful induction of tolerance, even though graft function may have been compromised and limited attempts were made to look for chronic rejection. A second problem relates to the difficulties in defining and assessing a state of immunological tolerance. Although in vitro assays of T-cell proliferation and cytotoxicity reflect MHC-driven alloreactivity, there are examples of T cells from animals with long-term, normally functioning allografts generating highly reactive MLR and CML responses in vitro (101). Findings such as these negate the use of these in vitro assays to accurately reflect a state of tolerance in vivo. The placement of donor-specific versus third-party skin grafts may also yield misleading information, since tissue-specific antigens play an important role in allograft rejection (102). Clearly, there is an ongoing need for new outcomes measures to detect and monitor the status of immune tolerance (103).

4.3. Central tolerance and cardiac allograft vasculopathy

In its strictest sense, the induction of central tolerance is analogous to self-tolerance, in which T-cells with high affinity for donor antigen are induced to undergo apoptosis through thymic-dependent mechanisms. Perhaps the most widely studied approach to central tolerance is through bone marrow transplantation and the induction of mixed hematopoietic chimerism (86) (104). It is generally felt that the tolerance that develops in these mixed chimeras is due to the migration of appropriate donor bone marrow elements (possibly dendritic cells) to the host thymus where they participate in clonal deletion of donor-reactive T cell precursors by negative selection (105).

Using a nonmyeloablative conditioning regimen prior to simultaneous donor bone marrow and kidney transplantation, Cosimi's group have demonstrated long-term renal allograft survival in MHC-mismatched non-human primates (106-108). Donor bone marrow engraftment in these animals resulted in multilineage chimerism detectable by flow cytometry. This peripheral macrochimerism typically fell to undetectable levels by 30-60 days after transplantation, but long-term survival of well-functioning kidney allografts was observed for as long as six years without chronic immunosuppressive therapy. These long-term survivors showed donor-specific unresponsiveness *in vitro*, never generated alloantibodies, accepted donor but not third party skin grafts and developed no signs of chronic rejection (108). This central tolerance strategy was recently applied to a patient with multiple myeloma and end-stage renal disease who, following myeloablative chemotherapy, received a therapeutic bone marrow transplant and subsequently a kidney from the her HLA-identical sister (109). Over two years later, she remains in remission with good renal function and no evidence of chronic rejection even though she is taking no immunosuppressive medication (T. R. Spitzer, personal communication).

Despite the success of mixed chimerism in seemingly preventing the manifestations of chronic rejection in renal transplant recipients, the same protocols may not be as effective in other organs. We have recently extended the mixed chimerism protocol to cynomolgus monkeys transplanted with MHC disparate cardiac allografts. After receiving the identical nonmyeloablative conditioning regimen and donor bone marrow infusion as the renal allograft recipients described above, three of five heart recipients developed donor marrow engraftment as indicated by chimerism in both the myeloid and lymphoid lineages. Like kidney recipients, donor macrochimerism in the heart recipients fell to undetectable levels 30-60 days after transplantation. Two of three heart recipients that developed multilineage chimerism survived more than one year with evidence of donor specific unresponsiveness in MLR and CML. However, in contrast to kidney recipients, all of the heart recipients developed anti-donor cellular and humoral immunity after chimerism disappeared and went on to reject their allografts in a chronic fashion with evidence of vasculopathy on histological examination (63).

In a separate study, MHC inbred miniature swine were T cell depleted using a porcine CD3 immunotoxin, and received nonmyeloablative preparative regimens and donor leukocyte transfusions to establish stable mixed hematopoietic chimerism across MHC-matched, minor antigen mismatched histocompatibility barriers (110). Hearts transplanted into the porcine mixed chimeras never rejected and the recipients were kept alive without immunosuppression, for over a year in some cases. However, when the hearts were eventually explanted, vascular lesions were detected in each allograft despite the lack of any interstitial rejection. Although few in number, these lesions produced luminal occlusion that was hemodynamically significant and which could have resulted in graft loss were the organ in a life-supporting position.

Finally, in a recent study, Russell *et al.* (56) explored the effects of tolerance on the formation of arteriopathy in transplanted mouse hearts. Specific tolerance was induced either by neonatal administration of allogeneic spleen cells (from F1 donors between class I-mismatched donor and recipient strains) resulting in "classical" immunological tolerance, or by bone marrow infusion to suitably prepared adult recipients, either fully MHC mismatched or class I mismatched, yielding "mixed chimerism." In both groups, donor-specific skin grafts survived perfectly, donor cell chimerism persisted, and alloantibodies were undetectable in all recipients. However, most transplants to either group of tolerant recipients

developed striking vasculopathy in their coronary arteries (12 of 15 in neonatal tolerance and 15 of 23 in mixed chimeras), while only 2 of 29 contemporary isotransplants showed any evidence of vasculopathy. Allografts transplanted into recipients essentially incapable of T and B cell responses (C.B-17/SCID and $RAG1^{-/-}$) also developed vasculopathy in 16 of 31 instances.

In summary, the development of cardiac allograft vasculopathy in the cynomolgus monkeys was not surprising given that a state of stable chimerism and tolerance was not maintained. However, the manifestation of chronic rejection in recipients demonstrating stable mixed chimerism and tolerance is harder to explain. Susceptibility to chronic rejection in recipients rendered tolerant by central deletional mechanisms may relate to the fact that, in its purest form, a state of central deletional tolerance induced by mixed chimerism is associated with certain immunological "blindspots" (111). For example, if tissue-specific antigens are expressed on the endothelium of donor coronary arteries but not on donor lymphohematopoietic cells, T cells reactive for these organ-specific alloantigens would not be deleted in the thymus of mixed chimeras and a smoldering immune response could result, leading to vasculopathy. Also, a purely deletional tolerance may not be effective in inducing linked suppression, a phenomenon in which tolerant T helper cells induce tolerance to additional allodeterminants expressed on the same APC over time (112) (113). Finally, the effects of central deletional tolerance on components of the innate immune system, such as NK cells and macrophages, which can contribute to chronic rejection (51), is still unclear.

4.4. Peripheral tolerance and cardiac allograft vasculopathy

The induction of peripheral tolerance via the generation of regulatory T cells or anergy may overcome the contribution of epitope spreading, transplant-related autoimmunity or other blindspots in central tolerance to chronic rejection. Many protocols have attempted to induce peripheral tolerance through T cell anergy, the generation of regulatory T cells or both. Indeed, there is substantial data that costimulatory blockade using CTLA4Ig (a competitive inhibitor of CD28 which binds to both B7-1 and B7-2) can prevent the initiation of experimental chronic rejection (114) and interrupt its progression in non-fully allogeneic rat models (115). Interestingly, in a fully allogeneic mouse heart transplant model, the prevention of chronic vasculopathy required either continuous CTLA4Ig therapy or the concurrent administration of donor cells (116). However, other studies have demonstrated that lesions of chronic rejection can occur in recipients rendered tolerant through peripheral mechanisms.

Mottram *et al*. (60) demonstrated that donor-specific tolerance can be induced to cardiac allografts in mice treated with anti-CD4 mAb as shown by the acceptance of donor-type skin grafts and rejection of third-party skin grafts placed 80 days after heart transplantation. However, 100 days following heart transplantation, all long-surviving allografts showed diffuse and prominent vascular intimal proliferation (mean luminal occlusion of 47% by morphometric analysis). Analysis of cytokine gene profile in these hearts revealed evidence of ongoing immune reactivity, although less than that of untreated controls. Thus, lesions of CR developed in the face of apparent peripheral tolerance.

Shimizu and colleagues (61), transplanted wild type BALB/c hearts into H-2 disparate CD40L-deficient ($B6CD40L^{-/-}$) mice. These recipients developed allospecific tolerance to the donor haplotype as indicated by the fact that second set donor skin grafts engrafted well, whereas third-party skin grafts were vigorously rejected. Also, by MLR, splenocytes from $CD40L^{-/-}$ allograft recipients demonstrated allo-specific hyporesponsiveness. Nevertheless, allografts in $CD40L^{-/-}$ hosts developed significant graft arteriosclerosis by 8-12 weeks posttransplant. In a pattern similar to that seen by Mottram *et al.* (60), intragraft cytokine expression demonstrated reduced but ongoing immune reactivity in the $CD40L^{-/-}$ recipients. The authors proposed that early alloresponses, without CD40-CD40L costimulation, induced allospecific tolerance but may have trigger allo-independent mechanisms that ultimately resulted in graft vasculopathy.

Larsen *et al.* (83) studied the ability of costimulatory blockade to abort T-cell clonal expansion in vitro and in vivo, and to prolong survival of murine cardiac allografts. The authors utilized the CTLA4Ig fusion protein to block costimulation of CD28/B7, and the monoclonal antibody MR1 to block CD40/CD40L interactions. The survival of MHC mismatched cardiac allografts was prolonged by CTLA4Ig alone and MR1 alone, but neither agent prevented the development of vascular lesions. In contrast, hearts in mice treated with CTLA4Ig plus MR1 were free of arteriopathy at 60 days and donor specific skin was accepted for at least 50 days. However, no information was provided as to whether lesions eventually formed in the donor heart over longer periods or after skin graft. This is relevant because further observation revealed that only 50% of animals treated with this regimen developed permanent skin graft survival (117).

Perhaps the most effective experimental protocol for inducing peripheral tolerance has been achieved by combining of CTLA4Ig with anti-CD40L mAb and rapamycin (118). This protocol not only led to the indefinite survival of H-2 mismatched cardiac allografts in mice, but also permitted the long-term acceptance of donor skin grafts. Unfortunately, however, the histology of the explanted allografts was not examined for the presence vascular lesions.

Taken together, these findings suggest that in some cases, recipients rendered tolerant via peripheral mechanisms can still develop manifestations of cardiac allograft vasculopathy, while in other cases they remain free of lesions. The most robust form of peripheral tolerance may be induced through costimulatory blockade along with a strategy aimed at optimizing the activation-induced cell death of donor-reactive cells. However, it remains unclear whether this strategy will uniformly prevent chronic rejection.

4.5. Is tolerance enough to prevent cardiac allograft vasculopathy?

Why do robust forms of immune tolerance sometimes fail to prevent chronic rejection? As mentioned above, there may be blindspots in tolerance induction protocols that allow a smoldering low-grade immune response to persist. The most obvious would be the contribution of nonimmune factors. It has become clear that factors such as ischemia/reperfusion injury, brain death, cytomegalovirus and senescence can all contribute to the process of chronic rejection (reviewed in (8,41)). As suggested earlier, these factors are probably not completely devoid of an immune component. However, a tolerance state alone would not provide absolute protection against these antigen-independent stimuli. A second possible explanation relates to the role of the innate immunity and cytokine excess. Activated macrophages can promote antigen recognition, cytokine production, cytoadhesion, chemotaxis, and endothelial/smooth muscle proliferative responses, and have been strongly implicated in the pathogenesis of chronic rejection (reviewed in (51)). When comparing chronically rejecting allografts to stable allografts there may be 100-300 differentially expressed genes for cytokines and other mediators of inflammation (119). Of all these mediators, however, interferon-γ stands out as major stimulus to chronic rejection. Studies using interferon-γ knockout mice (53) and antibodies to interferon-γ (52) have demonstrated the central role of this cytokine in chronic vasculopathy. These results have been corroborated by the recent finding that interferon-γ can elicit arteriosclerosis in the absence of immunocytes *in vivo* (54). Since NK cells are major producers of interferon-γ, this component of the innate immune system may play a key role in chronic rejection. Again, effector mechanisms related to the innate immune system would not be completely eliminated by the induction of immune tolerance. Third, transplantation-induced *autoimmunity* may play a role in chronic rejection. Such autoimmunity may be specific to the transplanted organ, as in the de novo autoimmunity to cardiac myosin demonstrated by Fedoseyeva *et al.* (57) after heart transplantation in mice and humans. (120). Alternatively, organ nonspecific autoimmunity may contribute to the inflammatory process that results in chronic rejection (58). Finally, *indirect allorecognition and epitope spreading* appear to be pivotal in the pathogenesis of chronic rejection (26). Recipient T cells indirectly primed against a restricted repertoire of immunodominant peptides may mediate chronic rejection either by providing help for alloantibody formation and/or by promoting lymphokine secretion required for macrophage and cytotoxic T cell activity (reviewed in (121)). Clinical studies have demonstrated the persistence of donor-specific MHC allopeptide T cell reactivity in patients with chronically rejecting cardiac (24,27,122), renal (28), and lung allografts (123). Furthermore, the specificity of T cell responses to donor antigens changes during the progression of rejection (epitope spreading) as has been demonstrated in human allograft rejection (24,28,124). Activation of naïve CD4+ T cells through the recognition of new peptides/determinants could thwart tolerance induction by consistently expanding the host's anti-donor T cell repertoire.

5. PERSPECTIVE

A true state of tolerance implies a rejection-free state, both acute and chronic, in the absence of chronic immunosuppression. However, conflicting reports generated from both central and peripheral models of tolerance have raised the question of whether tolerance will be enough to cure chronic rejection. One could easily argue that these were not “true” tolerance models, and thus strategies that do not lead to normal graft function and prevention of chronic rejection should not be considered true tolerance strategies. Other possible explanations may be mechanistic in nature. For example, although T cell activation is the central and primary event in allograft rejection, partial clonal anergy or elimination may be sufficient to prevent acute rejection and prolong survival but not sufficient to prevent indolent destruction of the graft. A related issue is the possibility that tolerance strategies, which preferentially block the direct pathway of allorecognition, may leave the indirect pathway unchecked. Elucidating the basis for organ susceptibility to chronic rejection in recipients rendered tolerant will clearly require further study (125). Our current observations emphasize that protocols designed to induce tolerance may have blindspots that will need to be addressed even after tolerance is induced. The studies described above clearly indicate the importance of monitoring graft function and vessel morphology in all animals and humans subjected to tolerogenic therapies. Also, it is clear that tolerance protocols will not be directly transferable from one organ system to another and that the preclinical testing of tolerance protocols for human transplantation must proceed in an organ-specific manner (126). Finally, before any truly meaningful evaluations of tolerance can be made in humans, effective immune monitoring tools will need to be developed to assess the state of tolerance, allowing the safe withdrawal of immunosuppression.

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