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Predominant expression of the Th2 response in chronic cardiac allograft rejection

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Abstract Chronic rejection is the main cause of late allograft failure in patients. CD4+ T cells activated by indirect recognition of alloantigens are implicated in this rejection reaction. However, the type of T cell response (Th1 vs Th2) that contributes to chronic rejection has not been fully investigated. The purpose of this study is to examine whether chronic rejection is associated with a polarized T-cell response in a rat cardiac allograft model, where long-term graft survival is achieved by intrathymic immunomodulation with donor class I, RT1.Aa, allopeptides. All long-surviving allografts showed histological evidence of chronic rejection. Chronic rejection was associated with high levels of intragraft Th2 cytokines and the Th2-regulated alloantibodies. The Th2 response was systemic, since long-surviving allografts with chronic rejection had high levels of serum IL-10. The predominance of the Th2 cytokines demonstrates that the Th2 response was not sufficient for the prevention of chronic rejection in this model. The predominant expression of Th2 cytokines, together with the presence of Th2-regulated alloantibodies, suggests that the Th2 response may play a role in the development of chronic rejection.

Keywords Chronic rejection · Cardiac allograft · Th2 response · Intrathymic immunomodulation · Indirect recognition

Introduction

Chronic rejection is the major cause of long-term allograft failure. The main features of chronic rejection are arteriosclerosis, interstitial fibrosis, and a gradual decline of organ function. Although the etiology of chronic rejection is multifactorial, immune responses generated by the foreign histocompatibility antigens play the most critical role [8, 25]. Importantly, traditional immunosuppressive protocols, while effectively preventing acute rejection, do not prevent chronic rejection [6, 37, 40]. This is at least partly due to a lack of understanding of the type of immunosuppression necessary to prevent the alloreactive responses that precipitate chronic rejection. A better characterization of the immune responses that are involved in chronic rejection may allow the designing of effective therapies that will ameliorate this type of rejection.

T-cell response to allografts is dictated by two distinct pathways of recognition of foreign histocompatibility antigens: direct and indirect [3, 13, 37]. The direct pathway involves recognition of intact allogeneic major histocompatibility complex (MHC) molecules on the surface of donor antigen-presenting cells (APCs) and initiates vigorous immune responses that precipitate acute allograft rejection [21, 38]. The indirect pathway involves recognition of histocompatibility antigens as peptides presented by syngeneic MHC molecules on the

surface of either donor or recipient APCs [3, 13, 37]. There are accumulating data in the literature suggesting that indirect recognition plays a critical role in chronic rejection [6, 23, 42]. For example, Ciubotariu et al. [6] demonstrated a direct correlation between T-cell response to allopeptides (corresponding to the hypervariable regions of 32 HLA-DR alleles) and the development of coronary artery vasculopathy in a large population of cardiac allograft recipients. Similar data were reported by Vella et al. [42] in kidney allograft recipients experiencing chronic rejection. Direct experimental data for the role of indirect recognition in chronic allograft rejection were recently provided by Lee et al. [23], who demonstrated that pre-immunization of pigs with donor class-I allopeptides promoted the development of chronic cardiac allograft rejection.

Although there are definitive experimental data implicating indirect recognition in chronic rejection, the type of alloreactive responses generated by this pathway that produce the lesions of chronic rejection are not well investigated. CD4+ T cells can be divided into at least two distinct subsets, Th1 and Th2, based on their pattern of cytokine production and effector function [28]. The Th1 response has been associated with acute allograft rejection, whereas the Th2 response has been implicated in long-term allograft survival in several experimental settings [37]. For example, cytokines produced by Th2 cells, such as IL-4 and IL-10, are expressed in long-surviving allografts with minimal levels of Th1 cytokines, IL-2 and IFN-γ [5, 41]. It is unclear, however, if the Th2 response actively contributes to graft acceptance or is only the byproduct of complex immunological responses that lead to long-term survival. We have recently hypothesized that a Th2 response may actively promote the development of chronic rejection by regulating a complex array of molecular and cellular interactions [37]. Although direct experimental evidence supporting this contention is lacking, the role of Th2 cells in humoral immunity, and the importance of alloantibodies in the development of chronic rejection, are consistent with this hypothesis [7, 37]. Furthermore, a few recent studies produced experimental evidence indicating that immune deviation to the Th2 response is not sufficient for successful engraftment of allogeneic organs, and increased expression of the Th2 cytokine IL-4 within the graft is responsible for the development of transplant arteriosclerosis [2, 11].

The purpose of this study is to characterize the type of T-cell responses associated with chronic rejection. Cardiac allografts in the PVG.R8-to-PVG.1U rat-strain combination, which were disparate for one class-I MHC, RT1.Aa molecule, were used for this purpose. We previously demonstrated that intrathymic immune modulation with donor RT1.Aa allopeptides, under the cover of transient immunosuppression with anti-lymphocyte serum, induced hypo-responsiveness to donor but not third party allografts in this model. The majority of allografts (~75%) survived long term (> 100 days), whereas a small portion of the grafts (~25%) underwent either acute or delayed rejection. Intrathymic immunomodulation with donor peptides, however, was not sufficient to induce transplantation tolerance, since a significant number of long-surviving allografts showed evidence of chronic rejection [39]. We here analyze cardiac graft recipients with chronic, delayed, and acute rejection for intragraft and systemic expression of cytokines and alloantibody isotypes to determine whether these forms of rejection are associated with selected types of T-cell responses. Our results demonstrate that there is a hierarchy in the T-cell response associated with different types of allograft rejection: grafts with acute rejection predominantly expressed the Th1 cytokines; those with delayed rejection expressed a mixture of Th1 and Th2 cytokines, whereas grafts with chronic rejection primarily expressed the Th2 cytokines. Taken together, these data demonstrate that a Th2 response is not sufficient for the prevention of chronic rejection in this model. The possible role of the Th2 response in the development of chronic rejection is discussed.

Materials and methods

Animals

PVG.1U (RT1.AuBuDuCu) and PVG.R8 (RT1.AaBuDuCu) rats were purchased from Harlan Sprague–Dawley (Indianapolis, Ill., USA). All animals brought into the experimental colony were certified virus-free, and the colony was monitored regularly for accidental contamination with infectious diseases. Age-matched (8 to 16-week-old) male animals were used throughout this study. All research protocols and general animal care were approved by the Institutional Animal Care and Use Committee and conform to the "Principles of Laboratory Animal Care" (NIH publication No. 86-23, revised 1985).

Intrathymic immune modulation

Long-term survival of cardiac allografts was induced by intrathymic injection of graft recipients with three donor RT1.Aa peptides (0.3–1.0 mg/peptide), either individually or as a mixture of various peptide combinations, as previously described [26]. At the completion of this procedure, each rat was given 1 ml of rabbit anti-rat lymphocyte serum (ALS) (Accurate Chemical, Westbury, N.Y., USA) intraperitoneally. Rats that were intrathymically given either PBS or an unrelated peptide of 18 residues corresponding to the rat TCR Vβ1 CDR2 domain served as negative controls.

We performed intra-abdominal heterotopic cardiac grafting, using PVG.1U rats as recipients for PVG.R8 donor hearts 7 days after intrathymic injection as described previously [26]. Syngeneic PVG.1U heart grafts were used as controls. Ventricular contractions were assessed daily by palpation. Rejection was defined as the day heartbeat ceased.

Cytokine ELISA

We measured the levels of cytokines (IL-2, IL-4, IL-10 and IFN-γ) in the sera of graft recipients, using the Cytoscreen Immunoassay Kit according to the manufacturer's instructions (BioSource International, Camarillo, Calif., USA). Serum samples were collected from rat recipients at different time points after transplantation. Standards of known concentrations, various dilutions of sera samples, and cytokine-specific biotinylated secondary antibodies were added to 96-well microtiter plates that had been coated with primary antibodies directed at individual rat cytokines. After the samples had been incubated for 3 h at room temperature, the plates were washed thoroughly with washing buffer four times and the samples were incubated with 100 µl HRP-streptavidin/well for 30 min at room temperature. After the samples had been further washed four times, 100 µl of stabilized chromogen (tetramethylbenzidine) was added to each well and the samples were incubated for 30 min in the dark. The reaction was stopped by addition of a stop solution. The optical density values were detected at 450 nm by a multi-label counter (Victor, Wallac, Gaithersburg, Md., USA) and expressed as nanograms per milliliter based on values obtained from standards of known concentration.

Histology

Immunohistological changes were studied in allografts harvested from long-term survivors ($n=8$; >200 days), short-term survivors ($n=2$; 102 and 131 days), mid-term rejecters ($n=6$; 15–100 days), and acute rejecters ($n=4$; 5–7 days). Syngeneic grafts in na recipients ($n=5$; 90 days) and those in recipients intrathymically manipulated with allogeneic antigens served as controls ($n=2$; 182–183 days). At harvest, all grafts were washed with 0.9% NaCl and divided into three sections (basal, midsection, and apical). The mid-ventricular sections were further divided into two portions, one of which was embedded in O.C.T. compound (Tissue-Tek, Miles, Elkhart, Ind., USA) and frozen by immersion in an isopentane solution. The frozen blocks were stored at −70 °C for immunohistological analysis. The remaining portions of the graft were fixed in 10% buffered formalin solution for hematoxylin and eosin (H&E) and elastic staining for assessment of general pathological changes. For each graft, three sections (the atria and base of the ventricles, mid ventricles, and ventricular apex) were observed under a light microscope, and histological scores were estimated. We assessed the intimal proliferation scores by analyzing eight to ten vessels per graft. Histological changes were evaluated on the following categories: vascular intimal proliferation, perivascular infiltration, myocardial infiltration, myocardial fibrosis, vasculitis, myocardial necrosis, and interstitial hemorrhage. Histological changes were scored blind and graded for individual lesions on a scale consisting of 0 (none) 1+ (mild), 2+ (moderate), and 3+ (severe) [9].

We measured the numbers of infiltrates present in the myocardium by counting cell nuclei/field for each graft. Ten high-power fields (400×) were randomly counted for each heart graft. The numbers of infiltrates present on the fields were calculated and expressed as the average numbers of cells per high-power field.

Elastic staining was performed on all heart grafts in addition to routine H&E histological examination. An elastic stain kit was used in accordance with the manufacturer's instructions (Sigma Diagnostics, St. Louis, Mo., USA). Tissue sections were stained in hematoxylin–iodine–ferric chloride solution and differentiated by the use of a dilute ferric chloride solution. We used Van Gieson solution to stain for internal as well as external elastic membrane.

Immunohistochemistry

The deposition of alloantibodies and selected cytokines was examined by the use of standard immuno-peroxidase techniques [9]. Briefly, 5-µm cryostat sections were prepared from heart allografts from different groups. The cryostat sections were incubated with primary antibody for 60 min in a humidified chamber at room temperature. The primary antibodies used in this study included: mouse anti-rat IgG1 (clone RG-88, Sigma), mouse anti-rat IgG2a (clone R2A-2, Sigma), mouse anti-rat IgG2b (clone R2B-8, Sigma), mouse anti-rat IgG2c (clone MARG2c-3, Sigma), mouse anti-rat IgM (clone RTM-32, Sigma), mouse anti-rat IFN-γ (clone DB-1, Serotec, England), mouse anti-rat IL-4 (clone MRC OX-81, Serotec), mouse anti-rat IL-10 (clone A5–7, Pharmingen, San Diego, Calif., USA), and rabbit anti-rat TNF-α (Serotec). After three washes in PBS (pH 7.4), the section slides were stained with secondary antibodies, HRP-conjugated rabbit anti-mouse or HRP-conjugated swine anti-rabbit immunoglobulins (Dako, Carpinteria, Calif., USA), at 1:100 dilution in PBS for 30 min at room temperature. After final washes in PBS, tissue sections were incubated with DAB substrate for the localization of peroxidase activity (FAST DAB, Sigma). Tissue slides were counterstained with diluted hematoxylin. Sections of heart tissue from na PVG.R8 rats or syngeneic grafts served as controls. Immunostaining was assessed via a semi-quantitative scoring system ranging from negative (−), trace positive (+), to strong positive (+++).

Results

Intrathymic immune modulation with class-I allopeptides does not prevent chronic rejection

We previously demonstrated that administration of one to three RT1.Aa allopeptides to the thymus under the cover of immunosuppression with anti-lymphocyte serum 7 days before transplantation resulted in indefinite survival of more than 75% of PVG.R8 hearts transplanted into PVG.1U rats [26]. The remainder of the grafts had either acute (5–10 days) or delayed rejection (15–100 days). One-half of long-surviving allografts that were analyzed ~100 days post-transplantation revealed incidences of chronic rejection [39]. The lack of chronic rejection in the remaining allografts may be due to their analysis too early in the post-transplantation period rather than transplantation tolerance induced by intrathymic immune modulation. In this study, we histologically analyzed long-term allografts at later times post-transplantation for incidence of chronic rejection.

All long-term allografts ($n=8$) that were analyzed >200 days post-transplantation revealed mild-to-severe evidence of chronic rejection, including a mild degree of myocardial infiltration by mononuclear cells, mild myocardial fibrosis, and various degrees of vessel wall thickening, ranging from focal intimal thickening to total vascular obliteration (Fig. 1A and Table 1). These findings are consistent with our previous studies [39] and a recent study demonstrating that intrathymic immune modulation with allogeneic cells prevented acute but not chronic rejection [20]. Allografts with delayed rejection (15–82 days; $n=6$) showed dense mononuclear cell infil-

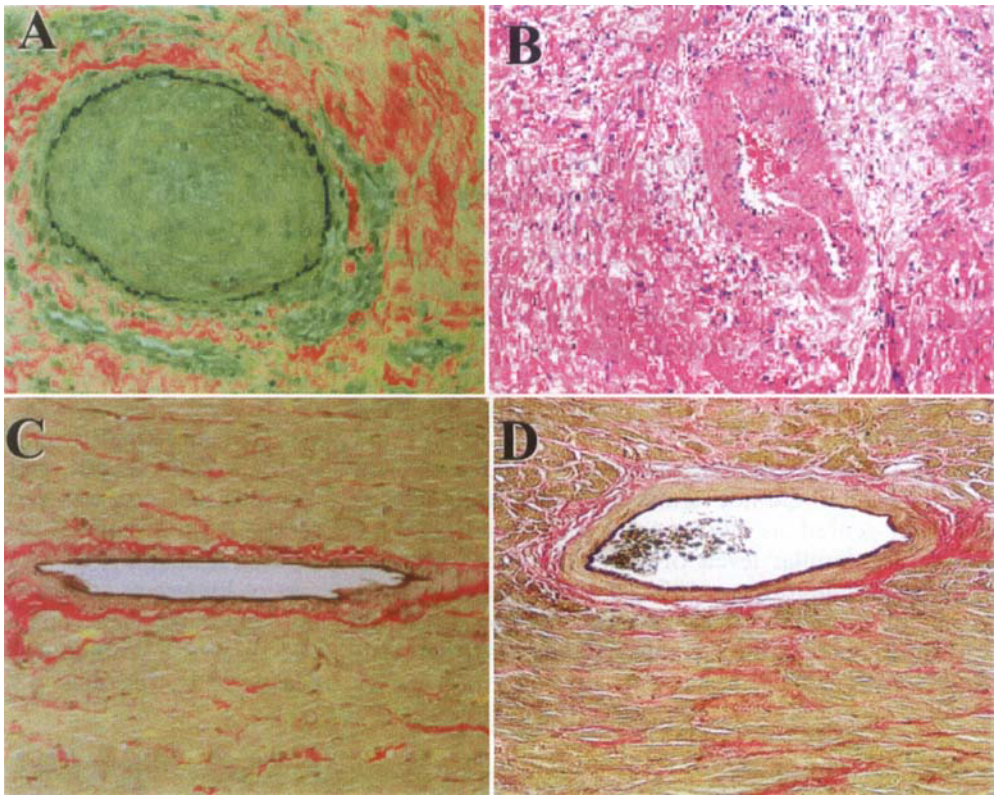

Fig. 1A–D Tissue sections from a long-surviving allograft (**A**), harvested at 300 days; an allograft with delayed rejection (**B**), harvested at rejection: 44 days; a syngeneic graft from a na recipient (**C**), harvested at 90 days; a syngeneic graft from a recipient intrathymically manipulated with alloantigen (**D**), harvested at 183 days. Unlike the allograft with delayed rejection (**B**) that shows severe mononuclear cell infiltration, interstitial edema, and necrosis, the allograft with chronic rejection (**A**) exhibited transplant vasculopathy caused by excessive hyperplasia in the intima (original magnification: ×200). Syngeneic grafts **C** and **D** did not show any sign of chronic rejection. Stains: H&E (**B**) and Elastic/Van Gieson (**A, C** and **D**)

Table 1 Histopathological scores. Syngeneicgrafts scored negative for all the indicated histological parameters. *PTx* post-transplant, *MI* myocardial infiltration, *PVI* perivascular infiltration, *MF* myocardial fibrosis, *MN* myocardial necrosis, *IH* interstitial hemorrhage, *IP* intimal proliferation, *Vas* vasculitis

Cardiac grafts	Harvest day PTx[b]	Histological score[a]						
		MI[b]	PVI	MF	MN	IH	IP	Vas
Long-term survival	300	368 ± 131[c]	1.0	2.0	0	0	1.0	0
	360	380 ± 158	1.0	0.5	0	0	0.5	0
	300	452 ± 155	0	2.0	0	0	2.0	0
	300	442 ± 139	0	1.0	0	0	0.5	0
	385	504 ± 106	1.0	2.0	0	0	2.0	0
	366	403 ± 147	1.0	1.0	0	0	0.5	0
Delayed rejection	15	724 ± 155	3.0	3.0	2.0	1.0	2.0	2.0
	23	754 ± 142	2.0	1.0	1.0	2.0	1.5	1.5
	44	765 ± 171	1.0	2.0	0	0	2.0	2.0
	63	768 ± 151	2.0	3.0	0	0	2.5	0
	79	576 ± 126	1.0	1.0	0	0	1.5	0
	82	424 ± 140	2.0	3.0	0	1.0	2.5	0

[a]Histopathological scoring ranged from 0 (none) to 3 (severe)
[b]Post-transplant
[c]Values represent mean number of cells ± SD per high-powerfield

tration, moderate-to-severe myocardial fibrosis, severe vascular proliferative lesions, and lesions related to acute episodes of allograft rejection, such as vasculitis, interstitial hemorrhage, and myocardial necrosis (Fig. 1B). In marked contrast to cardiac allografts with long-term survival or delayed rejection, syngeneic allografts lacked any sign of histopathological changes that were pertinent to chronic rejection (Fig. 1C and D), providing evidence for the importance of alloreactive immunity in the mediation of chronic rejection in this model.

Predominant expression of intragraft Th2 cytokines in chronic rejection

The nature of alloreactive responses that promote chronic allograft rejection has not been fully elucidated.

We previously hypothesized that the Th2 response may contribute to the initiation and/or maintenance of chronic rejection via its influence on humoral responses [37]. Cytokines elaborated by Th2 cells, such as IL-4, IL-5, and IL-6, serve as growth and differentiation factors for B cells [37]. We, therefore, investigated allografts with acute, delayed, and chronic rejection for intragraft and systemic cytokine expression at the protein level to test whether chronic rejection is associated with preferential activation of the Th2 response. Allografts with chronic rejection had high levels of Th2 cytokines, IL-4 and IL-10, and low-to-undetectable levels of Th1 cytokines, TNF-α and IFN-γ, as determined by immunohistological staining via antibodies specific for these cytokines (Fig. 2A). The expression levels of IL-4 and IL-10 in the long-surviving allografts (> 200 days) with chronic rejection did not significantly vary from graft to graft and scored as high (++) and moderate (+), respectively. Similar levels of expression were observed for IL-10 in allografts at 100–140 days post-transplantation. In contrast, IL-4 showed reduced levels of expression (+) in allografts analyzed 100–140 days post-transplantation when compared with those (++) for allografts at > 200 days post-transplantation. In marked contrast, allografts undergoing acute rejection (6 days) expressed high levels of TNF-α and IFN-γ and undetectable levels of IL-4 and IL-10 cytokines (data not shown). Allografts undergoing delayed rejection (15–82 days), on the other hand, expressed a mixed pattern of cytokines with high levels of Th1 and moderate levels of Th2 cytokines (Fig. 2B). Unlike allografts, syngeneic grafts had undetectable levels of all the cytokines examined.

High levels of systemic IL-10 in chronic rejection

We next investigated sera from recipients of allografts with chronic, delayed, and acute rejection to test if the cytokine response was systemic. There were high levels of IL-10 (> 10 nmol/l) in sera of graft recipients with chronic rejection (Fig. 3A). IL-10 was first detectable in sera of long-term survivors by day 14 post-transplantation, gradually increasing to peak levels on days 50–150, and showed a gradual decline thereafter (Fig. 3B). The decline in IL-10 levels in long-term allograft survivors,

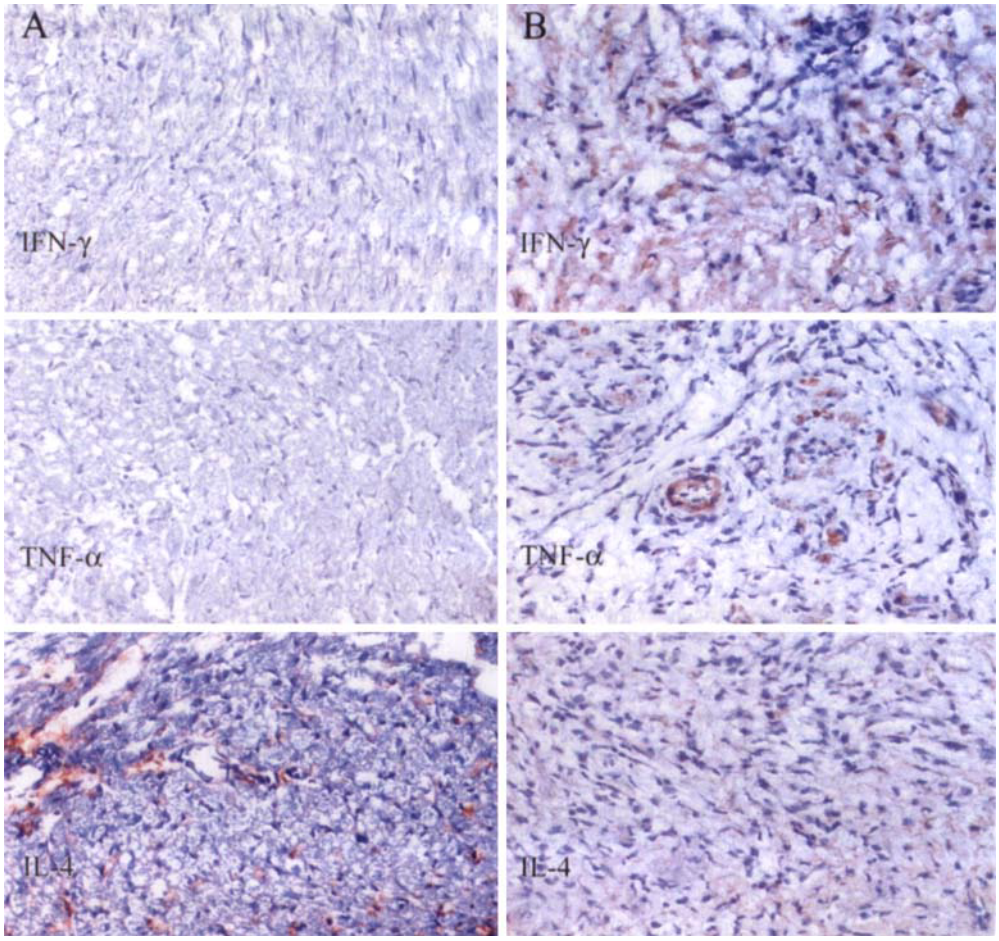

Fig. 2A, B Histology sections from allografts with chronic (**A**) and delayed (**B**) rejection. **A** is representative of grafts at > 200 days while **B** is representative of grafts at 35–82 days (original magnification: ×200). Allografts with chronic rejection expressed high levels of IL-4 and moderate to undetectable levels of IFN-γ and TNF-α. This pattern of cytokine expression was completely reversed in allografts with delayed rejection that expressed high levels of IFN-γ and TNF-α and moderate levels of IL-4

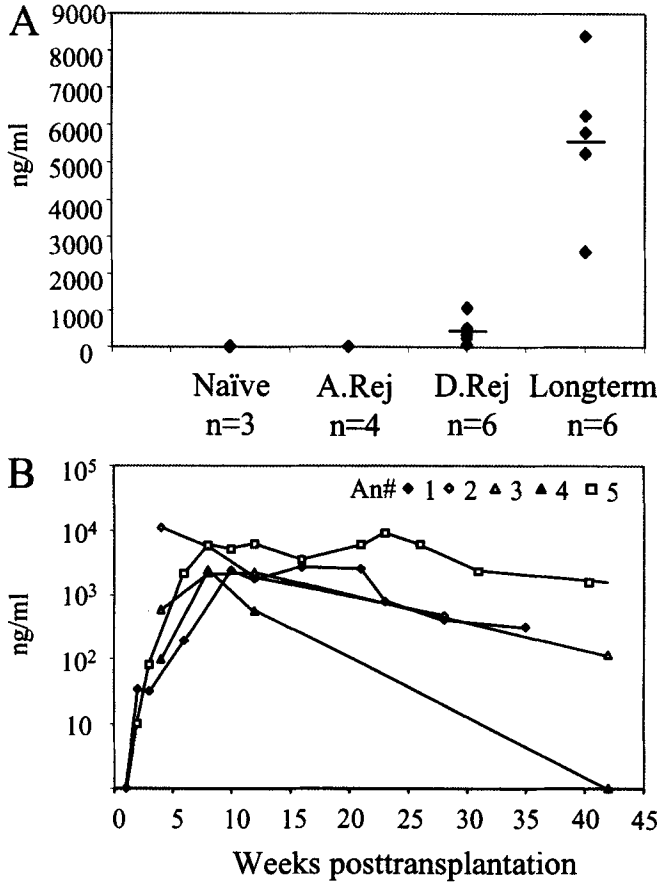

Fig. 3A, B Levels of IL-10. **A** Serum samples from graft recipients with acute rejection (*A.Rej*; 6 days post-transplantation), delayed rejection (*D.Rej*;15–82 days post-transplantation), and long-term survival (*Longterm*; >200 days post-transplantation), and from na rats that did not undergo transplantation (*Na*). **B** Serum samples from long-term survivors at various times after transplantation. The data are expressed as the mean of triplicate samples for each animal tested. The sensitivity of this assay is 39 pg/ml. *A#* animal number

however, did not reach background levels in more than 90% of graft recipients analyzed over 200 days post-transplantation. In marked contrast, sera from recipients with acute graft rejection had undetectable levels of IL-10. Graft recipients with delayed rejection had moderate levels of IL-10. Sera from na rats served as control with no detectable levels of IL-10. We did not detect systemic expression of IL-2, IL-4, and IFN-γ in recipients of allografts, regardless of the type of rejection reaction and the time of serum harvest (data not shown).

Alloantibody response in chronic rejection

Th2 cytokines are critical to the B-cell production of selected immunoglobulin isotypes such as IgG1 and IgG2a in the rat [15]. The predominant expression of Th2 cytokines in long-surviving allografts undergoing chronic rejection, therefore, led us to examine the intragraft deposition of alloantibodies, because the Th2 response is important to humoral immunity [29]. Additionally, alloantibodies have been implicated in the pathogenesis of chronic rejection in several experimental systems [7, 34, 36]. Long-surviving allografts had moderate intragraft deposition of IgG1, IgG2a, trace amounts of IgG2b, and no detectable levels of IgM and IgG2c antibodies (Fig. 4A). Allografts with delayed rejection harvested from intrathymically manipulated recipients had considerable deposition of IgM, IgG1, trace amounts of IgG2c, and relatively higher levels of IgG2a and IgG2b (Fig. 4B). Acutely rejecting allografts from unmanipulated control recipients had abundant levels of intragraft IgM, trace levels of IgG2a, and undetectable IgG1, IgG2b, and IgG2c isotypes. In contrast, syngeneic grafts had no detectable levels of any Ig subclasses examined (data not shown).

Discussion

The development of potent immunosuppressive agents has markedly reduced the loss of allografts from acute rejection. These immunosuppressive agents, however, are inadequate for averting late graft loss caused by chronic rejection. There is increasing evidence that indirect allo-recognition plays a critical role in chronic rejection [6, 23, 42]. However, the types of alloreactive responses generated by this pathway that precipitate chronic rejection are not well characterized. We have recently hypothesized that indirect recognition of allogeneic antigens late in transplantation may preferentially generate a Th2 response, and that this response may play a critical role in the pathogenesis of chronic rejection [37]. A series of experimental studies implicated a Th1 response in acute allograft rejection and a Th2 response in long-term allograft survival [37]. It is unclear, however, if the Th2 response actively contributes to graft acceptance or is only the byproduct of complex immunological responses that lead to long-term survival. Inasmuch as the Th2 response is important for humoral immunity and alloantibodies play a critical role in chronic rejection [7], the Th2 response may act to promote the development of chronic rejection rather than induce/maintain transplantation tolerance.

We herein demonstrated that chronic rejection in our model was associated with high levels of Th2 cytokines, IL-4 and IL-10, and moderate-to-undetectable levels of Th1 cytokines, TNF-α and IFN-γ. In marked contrast, allograft recipients with acute rejection had only Th1 cytokines. Intrathymically manipulated graft recipients with delayed rejection had features of both acute and chronic rejection. Moreover, they showed a mixed

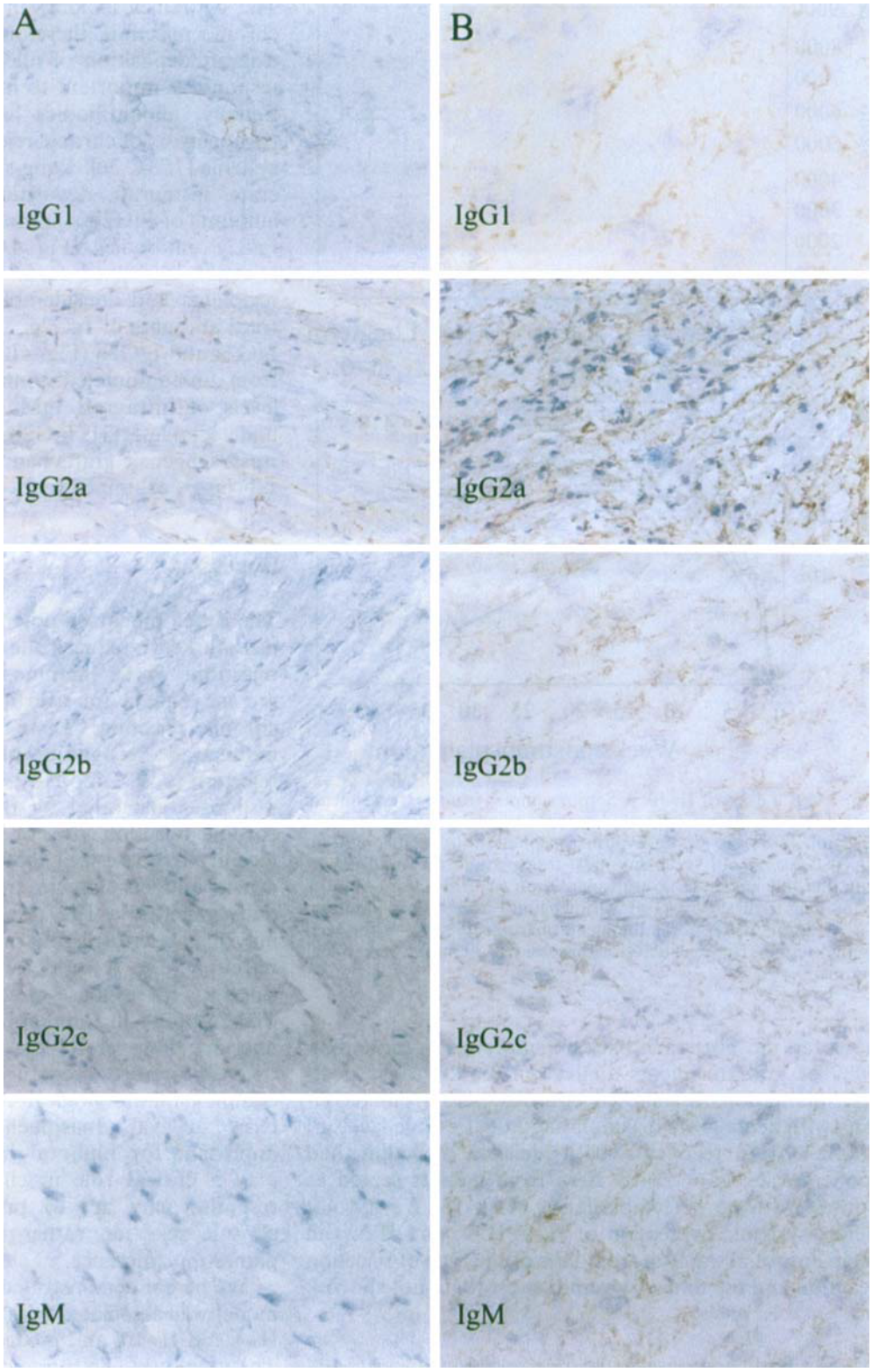

Fig. 4A, B Antibody deposition in cardiac allografts with acute and chronic rejection. Tissue sections were harvested from allografts with chronic rejection (**A**, representative of grafts at >200 days) and delayed rejection (**B**, representative of grafts at 35–82 days). Original magnification: ×200. Allografts with delayed rejection revealed the deposition of all the Ig isotypes examined, whereas allografts with chronic rejection primarily showed moderate deposition of IgG1 and IgG2a with undetectable levels of IgM, IgG2b and IgG2c isotypes

pattern of cytokine expression, predominated by the Th1 cytokines, IFN-γ and TNF-α. It is, therefore, tempting for us to speculate that the high levels of expression of pro-inflammatory IFN-γ and TNF-α cytokines, compared with the Th2 cytokines in the grafts with delayed rejection, may eventually shift the immune balance

towards a Th1 response that precipitates delayed acute rejection.

The Th2 response was systemic, as evidenced by extremely high levels of IL-10 (in the nanomoles per liter range) in the sera of chronically rejecting animals. There was a direct correlation between the serum levels of IL-10 and long-term allograft survival. All the allograft recipients that displayed high levels of IL-10 early in transplantation had prolonged graft survival. In marked contrast, allograft recipients that did not systemically express IL-10 rejected their grafts acutely, and those with moderate systemic expression of IL-10 had delayed rejection. The systemic expression of IL-10 in this model is of great interest, because this cytokine is synthesized by the Th2 cells in rodents and has been shown to specifically inhibit inflammatory responses mediated by the Th1 cells by several molecular mechanisms. IL-10 downregulates the expression of MHC, transporters associated with antigen processing, and B7 molecules on antigen presenting cells [27, 35]. IL-10 inhibits the infiltration of dendritic cells to the site of inflammation and impairs their function to stimulate Th1 but not Th2 cells [30]. IL-10 also downregulates metalloproteinases by directly inhibiting their synthesis by smooth muscle cells and macrophages and by stimulating the synthesis of their inhibitors by macrophages [22]. The negative regulation of these enzymes results in extracellular matrix accumulation in the intima, and the development of transplant arteriosclerosis [19]. IL-10 may, therefore, serve as an immunoregulatory molecule to downregulate the Th1 response to prevent acute rejection early post-transplantation and regulate alloantibody production and extracellular matrix production late post-transplantation, thereby plausibly promoting chronic rejection. This notion is consistent with a recent study demonstrating that exogenous IL-10 administration in mice leads to exacerbated allograft arteriosclerosis [14].

IL-10 regulates B cells for differentiation and production of antibody [33]. Alloantibodies have been implicated in the pathogenesis of chronic rejection in experimental as well as clinical settings [10, 32, 34, 36]. In animal experiments, direct evidence for the involvement of alloantibodies in chronic rejection was provided by studies in which B cell-deficient mice were used as graft recipients. Transplant vascular arteriosclerosis was shown to be significantly reduced in arterial transplants performed across various histocompatibility differences when IgM knockout mice that lack functional B cells were used as allograft recipients [36]. Similarly, the adoptive transfer of alloimmune serum to graft recipients that lacking an alloantibody response results in the development of the lesions of chronic rejection [10, 17, 34]. Alloantibodies may manifest their effect by cross-linking class-I molecules on the surface of endothelial cells and smooth muscle cells in the allograft, and by doing so, activate these cells for the synthesis of growth factors and receptors required for smooth muscle cell migration and proliferation in the intima [17, 18]. Consistent with this hypothesis is our demonstration of the deposition of IgG1 and IgG2a antibody isotypes in chronically rejecting allografts. These isotypes have been shown to be regulated by Th2 cytokines in the rat [15, 31]. Our data are also consistent with a study demonstrating that intrathymic immunomodulation with donor splenocytes results in intragraft deposition of IgG1 and IgG2a antibodies in a rat allograft model [4]. Interestingly, allografts in a similar rat intrathymic immune modulation model were recently shown to undergo chronic rejection [20].

Our data demonstrate that intrathymic immune modulation with class-I allopeptides preferentially induces intragraft and systemic expression of Th2 cytokines associated with long-term allograft survival. A Th2 response in this model, however, does not prevent chronic rejection, since long-surviving allografts showed extensive evidence of chronic rejection. Our findings, therefore, suggest that the Th2 response may play a critical role in the prevention of acute allograft rejection by down-regulating the Th1 response, but this response in itself is not sufficient to prevent chronic allograft rejection. Indeed, the Th2 response may initiate and/or perpetuate the process of chronic rejection by regulating the production of specific alloantibodies. This contention is consistent with several recent observations that long-term cardiac graft survival in a hamster-to-rat xenogeneic system induced by cyclosporin-A treatment is associated with the predominant expression of the Th2 cytokines and anti-apoptotic genes [1, 24]. These xenogeneic grafts, like the allografts described in this report, displayed extensive evidence of chronic rejection. Of direct relevance to our observations is a recent study demonstrating that treatment of graft recipients with antibodies to TCR or CD80/86 results in long-term graft survival in a murine cardiac allograft model [16]. Grafts in the TCR group developed chronic rejection, with high levels of expression of the Th2 cytokines. In marked contrast, allografts in the CD80/86 group did not develop chronic rejection and lacked the expression of Th2 cytokines. Direct evidence for the involvement of the Th2 response in chronic rejection was recently provided by two different studies performed in murine models. Chronic rejection of aortic allografts was prevented by the treatment of graft recipients with antibodies against IL-4 [12]. In another model, the administration of IL-10 to the recipients of heart allografts resulted in increased numbers of Th2 cytokines and accelerated chronic rejection [14].

We have previously demonstrated that the long-term graft survivors in this model had detectable levels of donor microchimerism in several tissues (the heart, kidney, liver, skin, bone marrow, thymus, and lymph nodes), using primers specific for the donor class-I

RT1.Aa gene in PCR [39]. Although there were no clinical signs of graft versus host disease (GvHD), it is quite likely that the long-term graft recipients may have developed chronic GvHD, since the animals were not assessed for histopathological changes related to GvHD. Therefore, the Th2 response observed in this model might be a reflection of graft-versus-host, rather than host-versus-graft, reactions. Further experimentation is needed if one is to address this possibility.

In summary, these observations are consistent with our recent hypothesis that a Th2 response may be important to the initiation/maintenance of chronic rejection [37]. The Th2 response may work in parallel with activated endothelial cells and B cells in the graft to facilitate the synthesis and elaboration of alloantibodies and a series of growth hormones, cytokines, and their receptors on smooth muscle cells to drive the proliferation of these cells in the intima and stimulate the development of graft vasculopathy. However, our data do not provide direct support for the role of the Th2 response in chronic rejection. The Th2 response observed in this model may simply be a byproduct of immunological mechanisms that precipitate chronic rejection, rather than being the cause of chronic rejection. Further studies in experimental and clinical settings are needed if the role of the Th2 response in chronic rejection is to be addressed.

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