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Superior 3-year kidney graft function in patients with impaired pretransplant Th2 responses

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Abstract In a prospective study of 80 patients, we investigated the association of kidney graft rejection with pretransplant CD4 helper/suppressor function, B cell responses, and in vitro cytokine secretion. A pokeweed mitogen-driven allogeneic coculture system was used to assess CD4 helper/suppressor function and T cell-dependent B cell responses. SAC I was used for T cell- and monocyte-independent stimulation of B cell cultures. B cell differentiation was assessed in a reverse hemolytic plaque assay. ELISAs were used to determine in vitro cytokine secretion. None of the 12 patients with pretransplant CD4 helper defects (< 10% helper activity) had acute rejection episodes in contrast to 32 of 68 (47%) patients with normal pretransplant CD4 helper function ($P = 0.001$). Patients with pretransplant CD4 helper defects exhibited better 3-year graft function than patients without CD4 helper defects (serum creatinine of functioning grafts: 1.2 ± 0.1 mg/dl compared to 1.7 ± 0.1 mg/dl, $P < 0.05$). Low pretransplant IL-10 responses (< 100 pg/ml; 14/80 patients) were significantly associated with a low incidence of acute rejection episodes ($P < 0.01$) and good 3-year graft function ($P < 0.05$). These data show that impaired pretransplant Th2 responses–CD4 help and IL-10 responses–predict a low risk of kidney graft rejection and good 3-year graft function, whereas Th1 (IL-2, IFN-γ) and B cell/monocyte responses are not of predictive value.

Key words Th2 · CD4 help · IL-10 · Kidney transplantation · Acute/chronic rejection

Introduction

We have shown previously that the risk of early acute rejection can be predicted by assessment of pretransplant, in vitro B cell responses [35]. Since unseparated peripheral blood mononuclear cells were used in the in vitro assays, it remained unclear whether T helper/suppressor activity, B cell/monocyte responses, or cytokine regulation were responsible for the effect. According to the Th1/Th2 model, Th1 products, such as interleukin (IL)-2 and interferon (IFN)-γ, are believed to be associated with acute allograft rejection, whereas the Th2 cytokines IL-4 and IL-10 should inhibit allograft rejection or even induce tolerance [19, 21, 28, 30, 33]. IL-2 stimulates both T cell proliferation and activation of cytotoxic T cells and is strongly expressed in grafts undergoing acute rejection [2, 24]. IFN-γ induces macrophage activation, upregulation of MHC expression, and enhances the activation of cytotoxic T lymphocytes [10]. Proinflammatory monokines and chemokines, such as IL-1, tumor necrosis factor (TNF)-α, IL-6 or IL-8, may also play a role in the acute rejection process [12, 13, 17, 23]. The Th2 cytokine, IL-10, however, inhibits Th1 cytokine secretion, MHC class II expression on monocytes, and the expression of B7 costimulatory ligands on the surface of macrophages [3–5, 7, 31], and thus might inhibit allograft rejection.

In a prospective study of 80 patients, we investigated whether pretransplant T helper/suppressor function or pretransplant in vitro B cell/monocyte responses predict the risk of graft rejection and 3-year graft function. The predictive value of the pretransplant in vitro synthesis of the Th1 cytokines IL-2 and IFN-γ, the Th2 cytokine IL-10, and B cell/monocyte-derived cytokines [IL-6, IL-8, TNF-α, granulocyte/macrophage colony stimulating factor (GM-CSF)] was analyzed in the same culture system.

Materials and methods

Patients

Eighty patients who were transplanted at the Department of Urology, University of Heidelberg, were studied prospectively during a 1-year posttransplant follow up. Of these, 74 patients had been on chronic hemodialysis and 6 patients on continuous ambulatory peritoneal dialysis; 69 patients received a first, 10 a second, and 1 a third cadaver transplant. Immunosuppressive treatment consisted of cyclosporine A (CsA)/steroids in 38 patients, azathioprine/CsA/steroids in 38 patients, azathioprine/steroids in 3 patients, and azathioprine/CsA in 1 patient. Thirty healthy blood donors served as controls. Lymphocyte subsets were assessed in 250 healthy controls.

Rejection episodes

Acute rejection episodes were diagnosed by typical clinical signs, accompanied by a rise in serum creatinine, and by renal scintigraphy and sonography. Graft biopsies were performed in 19 cases.

Flow cytometric analysis

Lymphocyte subsets were determined by indirect immunofluorescence and laser flow cytometry, as described previously [37, 39]. The following monoclonal antibodies were used: OKT3 (CD3), OKT11 (CD2), OKT4-FITC (CD4), OKT8 (CD8), OKIa-1, OKM5 (CD36; all monoclonal antibodies from Ortho, Raritan, N.J., USA), and Leu18 (CD45RA; Becton-Dickinson, Sunnyvale, Calif., USA). Cells were analyzed using a Facscan flow cytometer (Becton-Dickinson).

Cell separations

Cell separations were performed as described previously [38, 39]. Briefly, peripheral blood mononuclear cells were separated into "whole" T and non-T cells (termed B cells) by the rosetting procedure. Whole T cells were separated further into CD4 cell-depleted and CD8 cell-depleted subpopulations (termed CD8 + and CD4 + subsets) by treatment with OKT4 or OKT8 monoclonal antibodies, respectively, and rabbit complement. The B cell-enriched populations contained 2–4% CD2 + cells, 24–37% CD19 + cells, and 43–67% OKIa-1 + cells. T cell-enriched subsets were 92–96% CD2 + and < 2% CD19 +. CD4 cell-enriched subsets were 73–84% CD4 + and ≤ 6% CD8 +. CD8 cell-enriched subsets contained 70–79% CD8 + and < 11% CD4 + cells.

Coculture conditions

Pokeweed mitogen (PWM)-stimulated allogeneic cocultures of control B cells with patient T cells, CD4 + or CD8 + T cell subsets, PWM-stimulated allogeneic cocultures of patient B and control T cells, and SAC I-stimulated B cell cultures were performed as described previously [36, 38, 39]. B cell differentiation was assessed in a reverse hemolytic plaque assay. T helper and suppressor activity and B cell functions were calculated from the results [immunoglobulin secreting cells (ISC)/10^6 B cells] of the following cocultures:

T cell or T subset helper activity:

$$\frac{B(C)+T(P)+PWM-B(C)+T(P)+M}{B(C)+T(C)+PWM-B(C)+T(C)+M}$$

T cell or T subset suppressor activity:

$$1-\frac{B(C)+T(C)+T(P)+PWM}{B(C)+T(C)+PWM}$$

PWM-stimulated B cell function:

$$\frac{B(P)+T(C)+PWM-B(P)+T(C)+M}{B(C)+T(C)+PWM-B(C)+T(C)+M}$$

SAC I-stimulated B cell function:

$$[B(P)+SAC\ I]-[B(P)+M]$$

where B(C), T(C) = B and T cells of a control; B(P), T(P) = patient B or T cells (or T cell subsets); M = culture medium. Values of < 10% helper function were defined as defective helper activity, values of > 10% suppression as suppressor activity.

Cytokine secretion

Frozen plasma and frozen supernatants of unstimulated and PWM-stimulated allogeneic T and B cell cocultures and of SAC I-stimulated B cell cultures were thawed and tested for cytokine content by ELISA. Double sandwich ELISAs R & D Systems) were used for testing of IL-2 (human IL-2 Quantikine; R & D Systems, Minneapolis, Minn., USA), IL-6 (human IL-6 Quantikine; R & D Systems), IL-8 (human IL-8 Quantikine; R & D Systems), TNF-α (human TNFα Quantikine; R & D Systems), IL-10 (human IL-10 Cytoscreen; BioSource International, Camarillo, Calif., USA), IFN-γ (human IFN-γ ELISA; Hycult Biotechnology, Uden, The Netherlands), and GM-CSF (GM-CSF ELISA; Medgenix, Fleurus, Belgium). Cytokine responses were calculated from the results of the PWM-stimulated cocultures and the SAC I-stimulated B cell cultures from which the cytokine response of the corresponding unstimulated cultures were subtracted. Monokine and B cell cytokine responses (IL-6, IL-8, TNF-α, GM-CSF) were assessed in the supernatants of unstimulated B cell/monocyte cultures as well as unstimulated and PWM-stimulated cocultures of control T with patient B cells (including monocytes). Supernatants of SAC I-stimulated B cell cultures were tested for B cell cytokine content (IL-6). T cell cytokine secretion (IL-2, IL-10, IFN-γ) was assessed in the supernatants of unstimulated and PWM-stimulated cocultures of control B with patient T cells.

T cells produced only between 1% and 19% of B cell/monocyte-derived IL-10 in the PWM-stimulated coculture system. Control B cells/monocytes secreted between 2040 and 2500 pg/ml IL-10 if normal T helper activity was provided. Therefore, a low IL-10 response (< 100 pg/ml) in a coculture of patient T with control B cells reflects reduced T cell help for B cell/monocyte IL-10 secretion.

Statistics

Data are expressed as mean ± SEM. The chi-squared test, Wilcoxon rank-sum test, Fisher's exact test, and Pearson correlation coefficient were used for statistical analysis.

Results

Eighty patients were tested for pretransplant T helper/suppressor function, in vitro B cell responses, and cytokine secretion. Nine patients died with functioning grafts within 3 years posttransplantation (four within the first year). Graft failure was observed in nine patients (five in the first year) due to non-immunological graft failure ($n = 4$), acute irreversible rejection ($n = 1$), recurrence of the original disease (focal sclerosis, $n = 1$), and chronic rejection ($n = 3$). Of the remaining 62 patients, complete 1-year follow-up was available in all patients and 3-year follow-up was available in 53 patients.

The 2-year graft survival rate was 89% (71/80 patients). Acute rejection occurred in 32 of 80 (40%) patients. As expected, 1-year graft function was significantly associated with the occurrence of acute rejection episodes ($P = 0.0001$; data not shown).

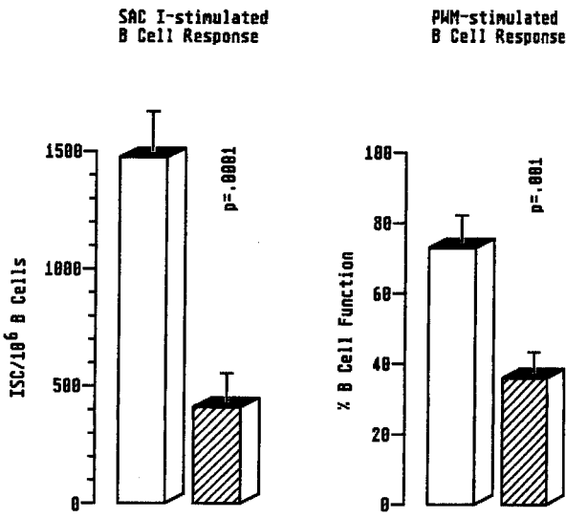

Fig. 1 In vitro B cell responses of SAC I-stimulated B cell cultures and pokeweed mitogen-stimulated allogeneic B cells with control T cell cultures in pretransplant patients and healthy controls. Results are given as mean ± SEM. *White columns* represent data of healthy controls and pretransplant patients are indicated by *striped columns*. *P* values for statistical comparison (Wilcoxon rank-sum test) between patients and controls (*ISC* immunoglobulin secreting cells)

Table 1 Plasma levels and in vitro pokeweed mitogen *(PWM)*-stimulated responses of B cell cytokines and monokines in pretransplant dialysis patients and healthy controls. Results are given as mean ± SEM (pg/ml). All patients were on chronic hemodialysis or continuous ambulatory peritoneal dialysis before transplantation. Wilcoxon rank-sum test was used for statistical *IL* interleukin, *TNF* tumor necrosis factor, *GM-CSF* granulocyte/macrophage colony stimulating factor, comparison between patients and controls (*NS* not significant)

		Patients ($n = 80$)	Controls ($n = 30$)	*P*
IL-6	Plasma	193 ± 60	115 ± 19	0.001
	SAC I-stimulated response	2204 ± 276	5369 ± 545	0.0001
	PWM-stimulated response	1960 ± 270	3672 ± 548	0.002
IL-8	Plasma	153 ± 58	76 ± 40	< 0.05
	PWM-stimulated response	128428 ± 27889	274752 ± 59022	< 0.005
TNF-α	Plasma	8 ± 2	2 ± 1	NS
	PWM-stimulated response	394 ± 90	235 ± 47	NS
GM-CSF	Plasma	0 ± 0	0 ± 0	NS
	PWM-stimulated response	3374 ± 386	5331 ± 504	< 0.01

Table 2 Incidence of acute rejection episodes and 3-year graft function in relation to pretransplant CD4 helper activity and IL-10 response in vitro. Results are given as mean ± SEM

	CD4 helper activity			IL-10 response		
	< 10%	≥ 10%	*P*	< 100 pg/ml	≥ 100 pg/ml	*P*
Incidence of acute rejection	0/12 (0%)	32/68 (47%)	= 0.001[a]	1/14 (7%)	31/66 (47%)	< 0.01[a]
Three-year serum creatinine (mg/dl)[c]	1.2 ± 0.1	1.7 ± 0.1	< 0.05[b]	1.3 ± 0.1	1.6 ± 0.1	< 0.05[b]
Immunological graft loss	0 patients	5 patients		0 patients	5 patients	

[a] Fisher's exact text
[b] Wilcoxon rank-sum test
[c] Serum creatinine values of functioning grafts

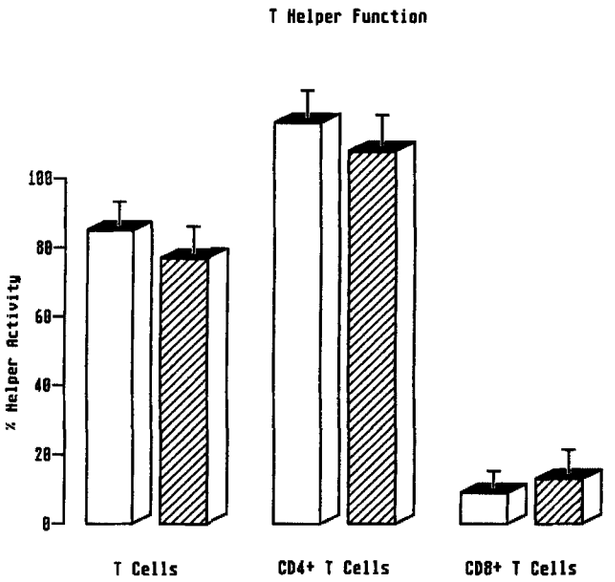

Fig. 2 Helper activity of "whole" T cells, CD4 +, and CD8 + T cells in pretransplant patients and healthy controls. Results are given as mean ± SEM. **White columns** represent data of healthy controls, *striped columns* those of pretransplant patients. Differences between patients and controls were not statistically significant

Pretransplant lymphocyte subsets

T cell counts were significantly reduced in pretransplant patients compared to healthy controls (T cells: 62 ± 2% in patients, 67 ± 1% in controls, $P < 0.005$; CD4 + T cells: 41 ± 1% in patients, 42 ± 1% in controls, not significant; CD8 + T cells: 22 ± 1% in patients, 27 ± 0% in controls, $P = 0.0001$). The counts of CD45RA + CD4 suppressor-inducer cells were comparable in patients and controls (50 ± 2% versus 52 ± 1%). They were not related to CD4 helper/suppressor function, incidence of acute graft rejection or 3-year graft function. Whereas monocyte counts were not significantly different, we found reduced B cell counts in the patient group (7 ± 1% in patients compared to 10 ± 0% in controls; $P = 0.0001$).

Pretransplant B cell/monocyte responses

Pretransplant mitogen-stimulated ISC responses were significantly impaired in patients compared to healthy controls (Fig. 1) and coincided with significantly reduced mitogen-stimulated B cell/monocyte-derived cytokine responses (IL-6, IL-8, GM-CSF; Table 1). Both ISC and cytokine responses were not associated with the incidence of acute rejection episodes or 3-year graft function.

Pretransplant T helper/suppressor function and cytokine responses

There was no significant difference in T helper/suppressor function when the mean values obtained in patients and healthy controls were compared (Fig. 2; data of T suppressor function not shown). However, helper defects of whole T cells and CD4 cells (helper activity < 10%) were found in individual patients but not in controls. It is an interesting finding that none of the 12 patients with pretransplant CD4 helper defects experienced acute graft rejection. In contrast, 47% of patients with normal pretransplant CD4 helper function had acute rejection episodes (Table 2). The increased

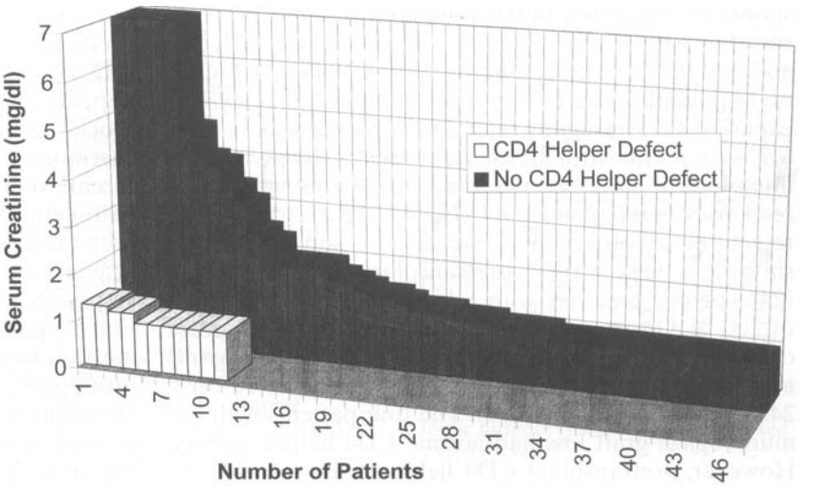

Fig. 3 Three-year serum creatinine values in patients with pretransplant CD4 helper defects and in patients with normal CD4 helper function. *Each box* represents one patient. Patients who lost their graft due to immunological reasons are represented with a serum creatinine level of 7 mg/dl ($n = 5$). Patients who lost their graft due to non-immunological reasons and patients who died with functioning grafts were not included. Pretransplant CD4 helper defects were significantly associated with good 3-year graft function ($P < 0.05$)

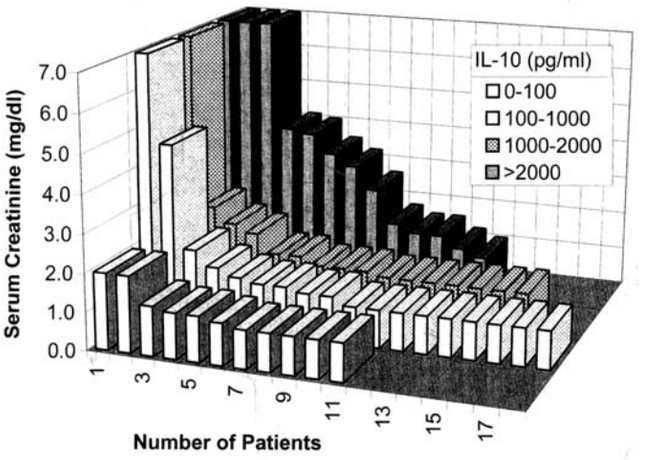

Fig. 4 Pretransplant in vitro IL-10 response and 3-year serum creatinine. *Each box* represents one patient. Patients who lost their graft due to immunological reasons are represented with a serum creatinine level of 7 mg/dl ($n = 5$). Patients who lost their graft due to non-immunological reasons and patients who died with functioning grafts are not included. One patient lost his graft due to recurrence of the original disease (IL-10 100–1000 pg/ml), one patient due to acute irreversible rejection (IL-10 > 2000 pg/ml), and three patients due to chronic rejection (IL-10 1000–2000 pg/ml, $n = 1$; > 2000 pg/ml, $n = 2$). Pretransplant IL-10 responses were significantly associated with 3-year graft function ($P < 0.05$)

incidence of acute rejections in the 68 patients coincided with impaired 3-year serum creatinine values (Table 2, Fig. 3). Furthermore, immunological graft loss was found in patients with normal pretransplant CD4 helper function only (five patients; Fig. 3).

Mitogen-stimulated secretion of IL-2, IL-10, and IFN-γ showed no significant differences between patients and controls. A significant difference in cytokine plasma levels was found for IL-2 (5 ± 1 pg/ml in patients versus 44 ± 6 pg/ml in controls; $P = 0.0001$).

It is impressive that a low pretransplant IL-10 response (< 100 pg/ml; 14/80 patients) was significantly associated with a low incidence of acute graft rejection and better 3-year graft function (Table 2, Fig. 4). Only 1 of 14 patients with low pretransplant IL-10 responses experienced acute graft rejection in contrast to 31 of 66 (47%) patients with IL-10 responses > 100 pg/ml. Immunological graft loss occurred in patients with IL-10 responses > 100 pg/ml only (Fig. 4). Acute irreversible rejection was observed in one patient, and this patient showed a very strong pretransplant IL-10 response (2446 pg/ml) together with a high CD4 helper activity of 200%. One patient lost his graft due to recurrence of the original disease (focal sclerosis; IL-10 855 pg/ml) and three patients due to chronic rejection (IL-10 1023, 2438, and 2466 pg/ml; Fig. 4). The four patients with immunological graft loss had normal CD4 helper activity. However, pretransplant CD4 helper activity and IL-10 responses were not significantly associated: 2 of the 14 patients with pretransplant IL-10 responses of < 100 pg/ml had CD4 helper defects, and only 2 of the 12 patients with CD4 helper defects had IL-10 responses < 100 pg/ml.

Discussion

Pretransplant in vitro B cell responses of unseparated peripheral blood mononuclear cells predict the risk of early acute allograft rejection [35]. In the current study, we were able to demonstrate that this phenomenon is related to the pretransplant CD4 helper function. Even graft loss due to chronic rejection occurred only in patients with normal pretransplant CD4 helper function. Pretransplant suppressor activity of CD4 or CD8 cells was not of predictive value. These data suggest that intensive immunosuppressive therapy, such as quadruple drug induction therapy, is unnecessary in patients with pretransplant CD4 helper defects. These patients would appear to be good candidates for rapid steroid tapering and eventual steroid-free maintenance.

B cell/monocyte responses (ISC and cytokine responses) were significantly impaired in patients on dialysis compared to healthy controls. Therefore, it was not unexpected that pretransplant B cell/monocyte responses were not predictive of acute rejection or 3-year graft function.

The most interesting finding of this study was that low pretransplant IL-10 responses predict a low incidence of acute allograft rejection and improved 3-year graft function. Immunological graft failure due to recurrence of the original disease, acute irreversible rejection, and even chronic rejection occurred only in patients with normal CD4 helper function and IL-10 responses > 100 pg/ml only. Since IL-10 inhibits monocyte functions and Th1 responses [3–5, 7, 31], elevated levels of IL-10 might be expected to inhibit allograft rejection. However, because dendritic cells are suspected to be the predominant antigen-presenting cells in the allograft response, and since IL-10 does not inhibit dendritic cell-dependent activation of naive T cells, IL-10 may not block the initiation of an alloimmune response [16]. Furthermore, IL-10 acts as a potent B cell growth and differentiation factor, stimulates antibody-dependent cellular cytotoxicity and can thereby augment humoral immune responses against the graft [6, 15, 22, 29, 34]. It is also conceivable that IL-10-induced suppression of Th1 responses promotes other forms of cellular graft rejection triggered by IL-4, IL-7, IL-9 or IL-15 [1, 32], which may not be sufficiently inhibited by conventional immunosuppressive therapy. In line with this hypothesis is the observation of Qian et al. [26] who showed that post-transplant treatment with IL-10 accelerated cardiac allograft rejection in a mouse model. Similarly, treatment

of islet cell-transplanted mice with an IL-10-immunoglobulin fusion protein did not prolong allograft survival [40], and Merville et al. [18] found a preferential production of IL-10 by graft-infiltrating cells in renal allograft recipients with accelerated vascular rejection. These data, together with our results, suggest that it is not useful to induce IL-10 secretion, for example by gene transfer, in order to downregulate the Th1 response. The reason might be that Th1 responses are already effectively inhibited by conventional CsA-based immunosuppressive therapy. In accordance with this hypothesis, we found no significant association between pretransplant Th1 responses (IL-2, IFN-γ) and the risk of acute graft rejection or 3-year graft function. Several immunosuppressive regimens, including therapy with CsA, corticoids, and anti-CD4 monoclonal antibodies, appear to inhibit Th1 but spare Th2 cytokine production [8, 9, 20, 27]. It might therefore be useful to suppress Th2 responses – CD4 helper activity and IL-10 responses – in order to prolong graft survival.

Recent studies in a rat model of carotid artery allografts [11] and of viral IL-10 gene transfer in a murine cardiac allograft model (DeBruyne et al. reported in [25]) provide evidence that Th2 cytokines might also contribute to the development of chronic rejection. Larsen et al. [14] found that blockade of both the CD28-B7 and CD40L-CD40 pathways inhibited both Th1 and Th2 cytokines and ablated chronic rejection in a mouse cardiac allograft model, whereas chronic rejection occurred after inhibition of either type of the T cell cytokines. A role of Th2 cytokines in chronic rejection is also suggested by our present data, since graft loss due to chronic rejection occurred only in patients with elevated IL-10 responses (> 1000 pg/ml) and normal CD4 helper function. A low IL-10 response appears to be associated with a significantly reduced risk of acute and possibly chronic graft rejection.

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