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Cytokine mRNA expression in endomyocardial biopsies during acute rejection from human heart transplants

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SUMMARY

The immune response to an allograft is regulated by cytokines produced by cells infiltrating the allograft. However, the immunopathogenesis of allograft rejection is not completely understood. To investigate the role of cytokines after clinical heart transplantation, we analysed the expression of cytokine genes in sequentially taken endomyocardial biopsies (EMB) by using the reverse transcriptase-polymerase chain reaction (RT-PCR). We analysed 44 EMB from 11 recipients: 21 EMB before or during rejection, and 23 EMB without histological evidence of acute rejection. A strong correlation was found between IL-2 gene expression and histologically proved rejection (16/21 *versus* 1/23 without rejection, $P < 0{\cdot}001$; χ^2 test). Also, expression of IL-4 and IL-6 genes was more often found in EMB during rejection than in EMB without signs of rejection (IL-4, 62% *versus* 35%; and IL-6, 81% *versus* 39%, respectively). No relation with rejection or with immunological quiescence was observed for the presence of IL-10 gene transcripts. IL-10, but also IL-6 mRNA were detectable in donor heart tissue before transplantation (9/10). In contrast, IL-2 and IL-4 gene transcripts were absent in these samples. These differences could not be explained by the presence or absence of T cells, since the gene for the constant region of the β-chain (Cß) of the T cell receptor (TCR) not only was expressed in post-transplant EMB but also in pre-transplant donor heart tissue. Our results provide strong evidence that the immunoregulatory cytokines IL-2, IL-4 and IL-6 are important local regulators in the graft during acute rejection. The role of IL-10 in the immunologic response to the transplanted organ needs further investigation.

Keywords clinical heart transplantation rejection messenger RNA IL-2

INTRODUCTION

The interaction of immunocompetent cells with their targets causes changes in the steady-state expression of different cytokine genes. Cytokines are soluble mediators of communication, and it has become clear that these regulators can be secreted both by graft-infiltrating cells and by cells from graft tissue. Evidence is increasing that different immunologic situations after organ transplantation may be associated with specific patterns of cytokine gene expression, although discrepancies in the detection between mRNA for certain cytokines and the specific cytokine protein have been reported [1]. In case of rejection, cytokine gene expression is observed even before tissue damage, as reflected by histology, or functional impairment of the graft is detected. Intragraft studies using Northern blotting and *in situ* hybridization following experimental and clinical transplantation showed that allogeneic reactions may be associated not only with expression of IL-2, IL-4 and interferon-gamma (IFN-γ) [2,3] but also with gene expression of proinflammatory cytokines such as IL-1β, tumour necrosis factor-alpha (TNF-α) and IL-6 [4–6]. In contrast, IL-10 and probably IL-4 might play a major role in suppressing rejection and inflammatory responses [7,8]. Although useful, both Northern blotting and *in situ* hybridization require relatively large amounts of mRNA and are technically difficult. In contrast, the reverse transcriptase-polymerase chain reaction (RT-PCR) provides a sensitive and reproducible method for the analysis of expression of several cytokine genes in a small number of cells. Using this technique, local transcription of various interleukin mRNAs was found during clinically relevant rejection episodes. In human kidney transplants an association was found with IL-2 expression, while in liver transplants IL-5 seemed to be more important [9–11]. After clinical lung transplantation, cytokine gene analysis on bronchoalveolar lavage material showed that IL-4 gene expression is

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more prominent at the time of a cellular rejection period [12]. The situation after heart transplantation is less obvious. Studies in experimental animals, cynomolgus monkeys and mice demonstrated that IL-2, IFN-γ, IL-4 and IL-6 gene transcription was induced within the transplanted heart during the processes of rejection [13,14]. In contrast, in human cardiac allografts a correlation was found between histological rejection and expression of IL-6 and transforming growth factor-beta (TGF-β) genes [15,16], which could be the result of aspecific inflammation rather than a T cell-mediated specific allogeneic reaction.

In order to assess cytokine transcription patterns that might be related to cell-mediated acute rejection or immunological quiescence after clinical heart transplantation, we determined the presence of IL-2, IL-4, IL-6 and IL-10 gene transcripts in donor heart tissue and in endomyocardial biopsies (EMB) post-transplantation by RT-PCR. By the same methodology, expression of the constant region of the β chain (Cß) gene of the T cell receptor (TCR) was performed to analyse the expression of cytokine genes in relation to the presence of a T cell infiltrate. The results were correlated with histological findings on simultaneously taken EMB.

PATIENTS AND METHODS

Patients

We analysed 44 EMB from 11 heart transplant recipients who showed histological signs of rejection. All EMB were taken during the first year after transplantation before, during and after rejection. Donor heart tissue taken before transplantation served as control ($n = 10$). Patients received cyclosporin A and low-dose steroids only as maintenance immunosuppressive therapy. Rejection was histologically diagnosed in EMB by the criteria of the International Society for Heart and Lung Transplantation [17]: in brief, grade 0, no evidence for rejection; grade 1A, focal (perivascular or interstitial) infiltrate without necrosis; grade 1B, diffuse but sparse infiltrate without necrosis; grade 2, one focus only with aggressive infiltration and/or focal myocyte damage; grade 3A, diffuse inflammatory process with necrosis; grade 3B, diffuse inflammatory process with necrosis; grade 4, diffuse aggressive polymorphous ± infiltrate, ± oedema, ± haemorrhage, ± vasculitis, with necrosis. For this study, EMB were grouped into those without myocyte damage (grade 0 and 1, $n = 23$), and those with myocyte damage (grade 2 and 3, $n = 21$).

RNA extraction and transcription

Donor heart tissue and post-transplant EMB for RT-PCR were snap-frozen in liquid nitrogen and stored at −80°C until analysis. Total RNA was extracted from snap-frozen EMB samples by a modification of the guanidinium method as described by Chomczynski & Sacchi [18]. EMB tissue was homogenized in 500 µl 4 M guanidine thiocyanate in the presence of 10^4 mouse 3T3 cells (ATCC, Rockville, MD). These mouse cells act as a carrier to improve the yield of total RNA. The solution was extracted with phenol, phenol-chloroform-isomethylalcohol [25:24:1] and chloroform-isomethylalcohol [24:1]. Total RNA was precipitated with 600 µl 2-propanol and 35 µl 3 M sodium acetate pH 5·2 at −20°C for 18 h. Precipitates were pelleted at 10 000 *g* at 4°C and washed with 500 µl cold 80% ethanol. Air-dried pellets were resuspended in 50 µl diethylpolycarbonate-treated H_2O. Total RNA was denaturated for 5 min at 80°C and then chilled on ice. First strand cDNA synthesis was performed from 25 µl of the isolated RNA with 0·25 µg hexanucleotides (0·5 µg/µl; Promega Corporation, Madison, WI) and transcribed with 2·5 µl Moloney murine leukaemia virus (MMLV) reverse transcriptase (200 U/µl; GIBCO-BRL, Gaithersburg, MD) at 42°C for 90 min in a total volume of 50 µl. The reaction mixture contained 10 µl 5× MMLV-RT buffer (250 mM Tris-HCl pH 8·3, 15 mM $MgCl_2$, 375 mM KCl), 2·5 µl dNTP (10 mM), 0·5 µl of RNAsin (400 U/µl; Promega) and 5 µl DTT (0·1 M).

PCR amplification and Southern blot analysis

Sequence-specific primers (Clontech Labs, Palo Alto, CA) were used for amplification of the human cytokine genes. These primers were located next to splice sites, to be able to discriminate for RNA only. cDNA sample (5 µl) was added to 45 µl PCR mixture containing 10 mM Tris-HCl pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·2 mM of each dATP, dCTP, dTTP, dGTP, 2 U Taq DNA polymerase (Promega) and 50 pmol of 5′ and 3′ sequence-specific primers. Each reaction mixture was overlaid with 50 µl mineral oil (Sigma, St Louis, MO) before PCR reaction in a DNA thermal cycler (Biomed, thermocycler 60) under the following conditions. After a 5 min 94°C denaturation step, samples were subjected to 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. The last cycle was extended with 7 min. As positive controls for the cytokines we used cDNA isolated from HuT78 cells (ATCC; a human T cell line derived from a patient with Sezary syndrome), and Epstein–Barr virus (EBV) immortalized B cells. The presence of T cells was determined by amplification of the gene coding for the β chain constant region of the TCR as described by Hawes [19]. PCR primers detecting transcripts for the human keratin gene (sense primer 5′TGAAGATCCGTGACTGGTAC3′ and anti-sense primer 5′ATGTCGGCTTCCACACTCAT3′) were used as an internal control to confirm successful RNA extraction and cDNA amplification [20]. After amplification, 8 µl PCR product were electrophoresed through 2% agarose gel, transferred to a Hybond-N+ membrane (Amersham, Aylesbury, UK) by electroblotting, and hybridized with γ^{32}P-labelled probes which are located across the splice site (cytokines, Clontech Labs; and keratin, 5′TGTCCTTCTGCAGATTGACAATGCCCGTCT3′). Hybridization was detected by autoradiography.

RESULTS

We analysed 10 samples of pretransplant donor graft tissue and 44 post-transplant EMB, obtained from 11 recipients for alterations in expression of IL-2, IL-4, IL-6 and IL-10 genes in relation to histological diagnosis. Transcripts from the human keratin gene were amplified in all tested samples. mRNA extracted from the mouse 3T3 carrier cell line did not show expression of genes coding for human cytokines, nor for Cβ of the TCR or keratin.

Pretransplant donor cardiac tissue

Genes coding for IL-6 and IL-10 were expressed in most of the pretransplant samples, whereas IL-4 mRNA was never detectable, and IL-2 mRNA was present in one sample only (Table 1).

Table 1. Cytokine and TCR-Cβ chain gene expression in pretransplant donor heart tissue. Southern blot analysis of polymerase chain reaction (PCR)-amplified DNA

Donor	IL-2	IL-4	IL-6	IL-10	TCR-Cβ chain
I	–	–	+	–	+
II	–	–	+	+	+
III	–	–	+	+	–
IV	–	–	+	+	+
V	–	–	+	+	+
VI	+	–	+	+	+
VII	–	–	+	+	+
VIII	–	–	+	+	+
IX	–	–	–	+	–
X	–	–	+	+	+
+/total	1/10	0/10	9/10	9/10	8/10

–, No signal; +, positive expression.

Also Cβ mRNA of the TCR was successfully amplified in eight of the 10 pretransplant samples (Table 1), reflecting the presence of the T cells in donor cardiac tissue.

Cytokine gene expression versus *histologic evidence of rejection*
Typical results of the kinetics of cytokine gene expression in pretransplant and post-transplant EMB for one patient before, during and after acute cellular rejection are shown in Fig. 1. Results of sequential EMB from 11 patients are presented in Tables 2 and 3 for EMB without and with histological evidence of rejection, respectively.

The presence of IL-2 transcripts was characteristic for EMB taken at the time of rejection, as 16/21 EMB (76%) showed transcription of the IL-2 gene, while only 1/23 (4%) EMB in which no myocyte damage was seen expressed the IL-2 gene ($P < 0{\cdot}001$; χ^2 test, Tables 2 and 3). IL-2 mRNA transcripts were already detectable in EMB with histological rejection grade 2, preceding grade 3 EMB (Table 3). Before or during an acute rejection episode, IL-2 mRNA-positive EMB frequently showed coexpression both of IL-4 and IL-6 mRNA (10/16). Nevertheless, the presence of IL-4 gene transcripts did not discriminate between EMB with and those without histological signs of rejection (13/21, 62% *versus* 8/23, 35%; $P > 0{\cdot}05$: Tables 2 and 3). Neither was the presence of IL-6 mRNA solely indicative for episodes of rejection, since in these cases 17/21 EMB (82%) expressed the IL-6 gene compared with 9/23 EMB (39%) taken at the time of immunological quiescence (Tables 2 and 3). For IL-10 gene no difference in transcription was found between the two EMB groups, since IL-10 mRNA was present in all EMB.

The cytokine mRNA profiles of the subgroups of EMB without myocyte damage, i.e. rejection grades 0 and 1A, were comparable (Table 4). The mild immune response reflected in EMB by the presence of cellular infiltration but without myocardial injury, apparently did not lead to expression of the IL-2 gene in the 10 grade 1A EMB tested.

TCR-Cβ gene expression versus *histologic evidence of rejection*
The IL-2 gene expression seen in EMB with rejection is supposed to be transcribed by activated T cells only. Other

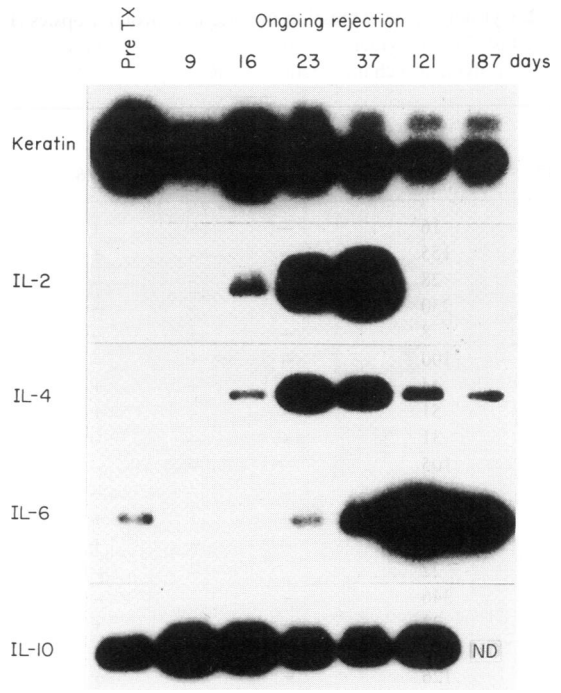

Fig. 1. Southern blot analysis of reverse transcriptase-polymerase chain reaction (RT-PCR)-amplified keratin, IL-2, IL-4, IL-6 and IL-10 transcripts from mRNA obtained from one patient (Me) of pretransplant and post-transplant endomyocardial biopsies (EMB), taken before, during and after rejection. Pre TX, Pretransplant EMB; day 9 post-transplant (pTX), no rejection; day 16 pTX, one focus only with aggressive infiltration and/or focal myocyte damage; day 23 pTX, one focus only with aggressive infiltration and/or focal myocyte damage; day 37 pTX, diffuse inflammatory process with necrosis; day 121 pTX, focal infiltrate without necrosis; day 187 pTX, no rejection.

cytokines may also be synthesized by cell types present concomitant in the graft. Therefore, we analysed the occurrence of T cells in the EMB in an attempt to enhance the discriminatory capacity of the other cytokine RT-PCR results. Although the Cß gene of the TCR appeared to be more prominently expressed in EMB with rejection than in those without, no significant correlation between the presence of T cells and the cytokine gene expression pattern could be made. Cβ gene transcription was detectable in all 44 post-transplantation EMB, those with and those without rejection, and even in pretransplant donor heart tissue (Fig. 2).

DISCUSSION

After transplantation, cytokines are transcribed and produced both by cells from graft tissue and by lymphocytes infiltrating the allograft. Cytokines mediate and control the allogeneic response, and therefore monitoring of cytokine gene patterns might be helpful in understanding immunological processes in the transplanted heart. Therefore, we determined cytokine and TCR-Cβ gene expression in pretransplant donor heart tissue and in post-transplant EMB.

We have shown that the intragraft cytokine gene pattern of post-transplant donor cardiac tissue differs from that of normal

Table 2. Cytokine gene expression in endomyocardial biopsies (EMB) without histological evidence of rejection*. Southern blot analysis of polymerase chain reaction (PCR)-amplified DNA

EMB†	Days post-transplant	IL-2	IL-4	IL-6	IL-10
Bi1	16	–	–	+	+
Bi4	155	–	–	+	ND
Bo1	28	–	–	–	+
Bo3	230	–	–	–	+
De1	8	–	+	–	+
De4	100	–	+	–	+
Ge2	44	–	+	+	ND
Ge3	51	–	–	+	ND
Ko1	31	–	–	–	ND
Ko3	105	–	–	–	ND
Me1	9	–	–	–	+
Me5	121	–	+	+	+
Me6	187	–	+	+	ND
Mi1	22	–	–	–	ND
Pe3	346	–	–	–	+
Po1	22	–	+	+	+
Po3	58	–	–	–	+
Po5	126	+	+	+	+
Se1	19	–	–	–	+
Se4	80	–	–	–	+
Se5	111	–	+	+	+
Se6	262	–	–	–	ND
Wa4	135	–	–	–	ND
+/total		1/23	8/23	9/23	14/14

* No rejection: EMB grading 0 and 1 according to Billingham *et al.* [17].

† EMB description: composed of patient identification and EMB number analysed.

ND, Not done; –, no signal; +, positive cytokine expression.

Table 3. Cytokine gene expression in endomyocardial biopsies (EMB) with histological evidence of rejection*. Southern blot analysis of polymerase chain reaction (PCR)-amplified DNA

EMB†	Days post-transplant	IL-2	IL-4	IL-6	IL-10
Bi2	60	–	+	–	+
Bi3	67	+	–	–	+
Bo2	125	+	+	+	ND
De2	29	+	+	+	+
De3‡	86	+	+	+	+
Ge1	16	+	–	+	ND
Ge4	72	+	–	+	ND
Ge5	101	+	–	+	ND
Ko2	99	–	–	+	ND
Me2‡	16	+	+	–	+
Me3‡	23	+	+	+	+
Me4	37	+	+	+	+
Pe1	59	+	+	+	+
Pe2	114	–	–	–	+
Po2	35	+	+	+	+
Po4	96	–	+	+	+
Se2‡	26	+	–	+	+
Se3	33	+	+	+	+
Wa1	16	+	+	+	ND
Wa2	23	–	–	+	ND
Wa3	108	+	+	+	ND
+/total		16/21	13/21	17/21	13/13

* Rejection: EMB grading ⩾ 2 according to Billingham [17].

† EMB description: composed of patient identification and EMB number analysed.

‡ Rejection grade 2 biopsies.

ND, Not done; –, no signal; +, positive cytokine expression.

heart tissue. We found that pretransplant donor heart tissue clearly contained IL-6 and IL-10 transcripts. In contrast, we hardly ever detected IL-2 and IL-4 gene expression in these samples. Therefore, the presence of these latter transcripts appeared to be characteristic of the immune response after transplantation. We found that local IL-2 mRNA transcription was correlated with cellular rejection leading to myocyte damage, after clinical heart transplantation. Moreover, analysis showed that IL-2 mRNA transcripts were already detectable in EMB histologically defined as rejection grade 2, in the period preceding rejection defined as rejection grade 3. Our results after clinical heart transplantation are in agreement with RT-PCR data after experimental heart transplantation [8,13,14,21]. The discrepancy between these data and those published by Zhao *et al.* [15,16] may be the result of differences in the amount of cardiac tissue from which the RNA was isolated, or in sensitivity of the RNA extraction. In contrast to the studies by Zhao *et al.*, we added carrier cells at the beginning of the RNA extraction which is necessary for optimal isolation from small amounts of tissue [22,23]. Also, their identification of a PCR product was solely based on the generation of a PCR product of predicted size, while the identity must be verified by a γ^{32}P-labelled internal probe, which is significantly more specific and sensitive. At this point one has to keep in mind that detection of mRNA using sensitive RT-PCR techniques may not give a correct impression of relevant production of the biologically active cytokine proteins. One could for instance hypothesize a (post)-translational blockade due to the clinical use of immunosuppressants, although such an activity of cyclosporin or prednisone has not been described. It would nevertheless be important for our understanding of the cytokine network in transplantation medicine if we could correlate mRNA expression with protein production. This, however, implies the introduction of an *in vitro* cell culturing phase with all its adherent confounding variables. The prominent place for IL-2 in the clinical rejection process as suggested by our RT-PCR results has also been found by Ruan *et al.* [24] in their immunohistochemical study. As in our present study, they showed that IL-2 was absent or only occasionally expressed in EMB without signs of myocyte damage. In our study, some of the tested EMB taken during rejection did not express the IL-2 gene. Apparently sampling timing is essential, and might be explained by the transient expression of IL-2 [25]. Besides, focal expression may cause discordance between EMB tested for cytokine gene expression and concurrently taken EMB obtained for histological evaluation.

Activated lymphocytes not only release IL-2 but also other cytokines such as IL-4 [26]. Like IL-2, IL-4 was also only

Table 4. Cytokine gene expression in the subgroups of endomyocardial biopsies (EMB) without myocyte damage

Gene product	Rejection grade 0 *n** (%†) (*n* = 13)	Rejection grade 1A *n* (%) (*n* = 10)
IL-2	1 (7)	0 (0)
IL-4	4 (31)	4 (40)
IL-6	5 (38)	4 (40)

* Number of positive EMB.
† Positive EMB as percentage of total EMB tested.

expressed in post-transplant EMB, but its presence had low predictive value for acute allograft rejection. Still, the finding that IL-2 and IL-4 genes are concurrently expressed during acute rejection indicates that not only IL-2 but also IL-4 play an important role in the regulation of the allogeneic response *in vivo*. This is in agreement with the observations by Dallman *et al.* [14] and by Morgan *et al.* [21], who reported that in non-immunosuppressed murine cardiac allografts genes for IL-2 and IL-4 are induced during rejection. The difference between the more clearcut role for IL-4 in these experimental studies and our less uniform findings might be the result of immunosuppressive therapy in the clinic. Also, recently published data of human lung transplant recipients demonstrated that induction of IL-4 gene transcription is far from uniform in allograft rejection [12].

In contrast to the mRNA for IL-2 and IL-4, the presence of the IL-6 and IL-10 mRNA was not confined to post-transplant EMB. The proinflammatory cytokine IL-6 and the anti-inflammatory cytokine IL-10 are not produced by activated T cells only [27,28], but are synthesized by a variety of cells, including monocytes [29,30] and endothelial cells [31,32] which are normally present in cardiac tissue. The inflammation that accompanies surgery would suffice to induce release of IL-6 [33] and possibly other cytokines such as IL-10. In the current study, measurement of IL-6 mRNA directly in post-transplant EMB was associated with, but again had no predictive value for, allograft rejection. IL-6 transcripts were present in 81% of the EMB with clinically relevant rejection (myocyte damage), and in only 40% of those with irrelevant rejection (infiltrate only). It is likely that IL-6 is involved in the inflammation process occurring during acute rejection, since the presence of IL-6 transcripts in post-transplant EMB is associated with a massive infiltration of T cells (Fig. 2) and monocytes [34] in the allograft, which suggests that at least some of the IL-6 gene expression is derived from cells invading the allograft. The positive correlation of IL-6 expression to acute renal graft rejection is supported by several studies [4,35,36]. This relationship, however, was not found after clinical liver transplantation, where intragraft IL-6 mRNA transcription was not associated with allograft rejection [10]. Interestingly, in the present study, the IL-10 cytokine gene was persistently expressed both before and after transplantation. Recently published data from mouse studies showed that this cytokine is transcribed not only during immunological tolerance, but also at the time of cellular rejection [8]. Furthermore, Merville *et al.* showed that a high proportion of cells derived from a human kidney with irreversible rejection secreted IL-10 [35]. Since IL-10 can both suppress the immune response [37–39] and act as a cofactor to promote cell growth [40], a continuous presence of mRNA for IL-10 under different immunological conditions can be explained. To clarify the exact relationship between IL-10 gene expression and T cell infiltration in the transplanted organ more precisely, a quantitative or semiquantitative analysis is required.

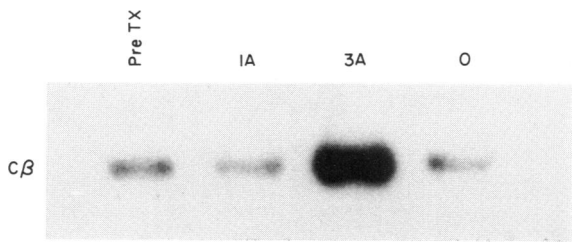

Fig. 2. Southern blot analysis of reverse transcriptase-polymerase chain reaction (RT-PCR)-amplified β chain transcripts of the constant region (Cβ) of the T cell receptor (TCR) from mRNA obtained from one patient (Ko) of pretransplant allograft tissue and post-transplant endomyocardial biopsies (EMB). Histology: pre TX, donor heart tissue before transplantation; day 31 post-transplant (pTX), 1A, focal (perivascular or interstitial) infiltrate without necrosis; day 99 pTX, 3A, diffuse inflammatory process with necrosis; day 105 pTX, 0, no rejection.

In conclusion, rejection and immunological quiescence were correlated with distinct patterns of cytokine gene expression. We found a clear correlation of IL-2 and to a lesser extent of IL-4 and IL-6 gene expression with myocyte damage. These cytokine profiles might be of prognostic significance for rejection processes after clinical heart transplantation. However, the role of IL-10 in processes leading to rejection or immunological tolerance remains to be determined.

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