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High frequency of IL-4 producing helper T lymphocytes associated with a reduced incidence of heart allograft rejection

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Abstract The reduction in the frequency of rejection episodes several months after heart transplantation (HTX) correlates with the development of donor-specific nonresponsiveness. This is reflected in a reduced frequency of donor-specific cytotoxic T cells (CTL) in the peripheral blood. We investigated whether the reduced CTL frequency and the incidence of rejection episodes coincided with a change in the frequency of either IL-2- or IL-4-producing helper T lymphocytes (HTL). We measured the frequency of HTL before and at several time points after HTX in the blood of ten recipients, using limiting dilution analysis for IL-2 and IL-4. In most patients, HTL frequencies dropped immediately after transplantation, but returned to pre-HTX values later after transplantation. No consistent decrease or increase in frequencies was observed long after HTX. In contrast to IL-2, the HTL frequencies for IL-4 before transplantation were significantly higher in patients without post-HTX rejection episodes requiring treatment than in patients with such episodes. This phenomenon was observed for the in vitro responses towards both donor and third-party cells. In conclusion, relatively high frequencies of IL-4-producing T cells may have a beneficial effect on the outcome of human heart transplantation, because they are associated with a reduced incidence of rejection episodes after transplantation.

Key words Heart transplantation · Limiting dilution analysis · pHTL · Interleukin-2 · Interleukin-4 · Nonresponsiveness

Introduction

Acute rejection of human allografts occurs most frequently during the first months after transplantation. In heart transplant recipients, hardly any acute rejection episodes are observed more than 1 year after transplantation. It was demonstrated that these patients develop a certain level of donor-specific nonresponsiveness, which is reflected in a reduced in vitro cytolytic reactivity towards the donor and a reduced frequency of cytotoxic T lymphocytes (CTL) in the peripheral blood of the patients [13, 37]. This reduction in donor reactivity develops spontaneously, but is not complete: although the dosage of immunosuppressant agents can often be lowered when the graft function is stable, most patients require life-long immunosuppression to maintain the graft. The mechanism of the development of nonresponsiveness is largely unknown.

In animal models, prolonged graft survival or donor-specific tolerance can be induced by different protocols.

Intervention with the T cell costimulatory pathway, using CTLA4-Ig, can induce prolonged heart or kidney graft survival in rodents and primates [16, 18]. In some mouse models, this treatment can induce tolerance to cardiac grafts [10, 17]. Another successful protocol involves treatment with depleting or nondepleting antibodies to CD4, in combination with a donor-specific transfusion, for example. This treatment can induce long-term graft survival across major histocompatibility barriers [3, 22]. In this model, $CD4^+$ T cells of the host play an important regulatory role in the induction of tolerance [3, 6, 23]. $CD4^+$ T cells can even transfer tolerance to naive animals [5]. Cytokines expressed during tolerance induction may mediate the suppression of donor reactivity. Especially IL-4 and perhaps IL-10, which can both be produced by T cells of the T helper 2 (Th2) phenotype, may play a part, by inhibiting the donor-reactive T cells of the Th1 phenotype. These T cells can produce IL-2 and IFNγ, and are believed to be involved in graft rejection. In some studies, IL-4 and IL-10 were indeed up-regulated in tolerant animals [10, 32]. Antibodies to IL-4 and IL-10 could partially reverse the suppressive effect of $CD4^+$ T cells during tolerance induction [6, 26]. However, tolerance could also be induced in IL-4 knockout mice, indicating that IL-4 may be important, but is not absolutely essential, for the induction of tolerance [17].

Donor-specific nonresponsiveness in human heart allograft recipients has been shown to be correlated with reduced CTL reactivity [13, 37]. This could be the result of suppression by regulatory $CD4^+$ helper T lymphocytes (HTL) and the production of regulatory cytokines. To evaluate whether IL-2 and IL-4 play a role in the induction of nonresponsiveness, we studied the frequency of IL-2 and IL-4 producing HTL in peripheral blood before and after heart transplantation (HTX), using limiting dilution analysis (LDA). We evaluated whether these frequencies were correlated with the incidence of rejection episodes after HTX, and whether a decrease in the occurrence of rejection episodes was reflected in a change in the frequency of IL-2 or IL-4 producing HTL. The development of nonresponsiveness appeared not to be correlated with a change in IL-2- or IL-4-producing HTL frequencies. However, we did observe that a high frequency of IL-4-producing HTL before transplantation correlated with a lower incidence of rejection episodes requiring additional immunosuppressive treatment.

Materials and methods

Patients

Ten patients who had each received a heart transplant between 1992 and 1995 were selected on the basis of HLA class II matching of donor and recipient. Five patients were chosen who in retrospective comparison with their donor were found to have HLA-DR mismatches for both alleles. These five patients, included four who also had one or two HLA-DQ mismatches. The other five patients shared at least one HLA-DR and one HLA-DQ allele with the donor. All patients were typed for HLA class I and class II by serology. For some patients, typing for HLA-DR was confirmed by PCR using sequence-specific primers. All HLA-types are summarized in Table 1. Standard immunosuppressive therapy and rejection therapy was given as described previously [13].

Rejection scores

Rejection episodes were diagnosed by histopathological examination of endomyocardial biopsies, according to the criteria of the International Society for Heart and Lung Transplantation [1]. For the ten patients, a rejection score was calculated, being the sum of all rejection episodes occurring during the first 1.5 years after (HTX). A grade 0 was scored as 0, grade 1 A or 1B as 1, grade 2 as 2, and grade 3A or 3B as 3. Rejection scores are listed in Table 1.

Mononuclear cell isolation from blood and spleen

Blood samples were drawn from the patients before and at various time points after HTX. Donor spleen tissue was homogenized in RPMI1640 until a cell suspension was obtained. Mononuclear cells (MNC) were isolated from blood and from the spleen cell suspension by Ficoll density gradient centrifugation. Although the number of $CD3^+$ T cells was affected by the immunosuppressive treatment during the first weeks, no major changes occurred in the CD4/CD8 ratio, as was reported previously [25]. The cells were cryopreserved overnight at –80 °C and stored in liquid nitrogen until needed.

LDA of HTL producing IL-2 and IL-4

LDA was used to determine the frequency of the population of alloantigen-specific IL-2- or IL-4-producing HTL [29].

Two-fold serial dilutions were made of responder MNC from patients at four time points: pre-HTX, and then 1–2 weeks, 3–5 months and 15–21 months post-HTX. Time points were chosen at which patients did not suffer from rejection episodes. The dilution series was made in 24-replicate wells of 96-well U-bottom plates, in RPMI1640, Dutch Modification, containing 10% heat-inactivated pooled human AB-serum (referred to as RPMI/10%AB). The series of responder cells ranged from 16×10^4 to 0.0156×10^4 cells/well in 100 µl RPMI/10%AB. B cells from donor spleen were EBV-transformed, and used as stimulator cells. Autologous EBV-transformed B cells were not included as controls, since it has been reported that in an allogeneic stimulation, the HTL frequency is independent of the EBV immune status of the responder [7]. Stimulator cells were irradiated (50 Gy) and were added to the responder cells at 5×10^4 cells/well in 50 µl RPMI/10%AB. To determine the background values, 24 wells containing only stimulator cells were included. As a control for donor specificity, a complete dilution series was run in parallel for each time point, in which donor stimulator cells were replaced by third-party EBV-transformed B cells. Third-party cells were completely mismatched to both patient and donor for at least HLA-B and DR, and if possible also for HLA-A, C and DQ (Table 1). The plates were incubated at 37 °C in 5% CO_2 for 64 h. Supernatants were harvested (50 µl/well) and transferred to new 96-well plates. For IL-2, supernatants were harvested from 2×10^4 to 0.0156×10^4 re-

Table 1 HLA types of patients, donors and third parties, total number of mismatches for HLA-A, -B and -DR, and rejection scores. HLA types in italics indicate similarities between patient and donor. Underlined HLA types indicate similarities between patient and third party. HLA subtypes are indicated between brackets (*n. d.* not determined)

Patient	HLA-A	HLA-B	HLA-DR	HLA-DQ	No. mismatch A, B, DR	Rejection score[a] rejection grades)
A Patient	3,19 (29)	15,12 (45)	4,6 (13)	1,3	6	2
Donor	1,2	8,5 (51)	1,3	n.d.		(1 × 2)
3rd party	19 (31), 19 (32)	35,39	5 (11),–	3,–		
B Patient	2,28	12,–	4,5 (11)	3,–	5	7
Donor	2,3	7,27	1,2	1,–		(1 × 3A, 1 × 2, 2 × 1A)
3rd party	2,9 (24)	42,22 (55)	6 (13),7	1,2		
C Patient	2,3	5 (51), 16 (39)	4,8	3,–	5	11
Donor	1,3	7,8	2 (15),–	1,–		(1 × 3A, 2 × 2, 4 × 1A)
3rd party	9 (23), 9 (24)	18,12 (44)	1,7	1,2		
D Patient	1,10 (26)	7,27	1,4	1,3	5	1
Donor	1,2	8,5 (51)	3 (17),6 (13)	1,2		(1 × 1B)
3rd party	19 (31), 19 (32)	35,39	5 (11),–	3,–		
E Patient	3,28	27,35	2 (15),6 (14)	1,–	4	8
Donor	1,3	8,35	3,5 (11)	2,3		(2 × 3A, 2 × 1A)
3rd party	9 (23), 9 (24)	18,12 (44)	1,7	1,2		
F Patient	1, 11	8,15 (62)	5 (11),3 (17)	2,3	3	11
Donor	1,2	8,13	3,7	2,–		(1 × 3B), 2 × 2, 4 × 1A/B)
3rd party	3,11	35,16 (39)	1,2 (16)	1,–		
G Patient	2,10 (26)	8,16 (39)	3,2 (15)	1,2	3	5
Donor	1,3	7,8	3,2 (15)	1,2		(1 × 2, 3 × 1A)
3rd party	9 (23), 9 (24)	18,12 (44)	1,7	1,2		
H Patient	2,9 (23)	7,21 (49)	8,2 (15)	1,3	3	5
Donor	3,–	7,14	6 (13),2 (15)	1 (6)		(1 × 3A, 2 × 1A)
3rd party	19 (29),–	12 (44),–	7,–	2,–		
I Patient	2,3	7,15 (62)	4,6 (13)	1,3	2	0
Donor	2,–	12 (44), 40 (60)	6 (13),–	1,–		
3rd party	19 (31), 19 (32)	35,39	5 (11),–	3,–		
J Patient	2,3	35,5 (51)	1,4	1,3	1	0
Donor	3,28	35,–	1,–	1,–		
3rd party	1,3	7,8	3,2 (15)	1,2		

[a] The rejection scores are calculated using the numbers and grades of rejection episodes (indicated between brackets). A grade 0 was scored as 0, grade 1 A or 1 B as 1, grade 2 as 2, and grade 3 A or 3 B as 3

sponder cells/well, and for IL-4 from 16×10^4 to 0.125×10^4 responder cells/well.

The amount of IL-2 in the supernatants was measured using the IL-2-dependent murine CTLL-2 cell line, which is sensitive to IL-2 but not to IL-4. For IL-4, the IL-4-dependent murine CT.h4 S cell line was used, which is sensitive to IL-4 but not IL-2, because it is transfected with the human IL-4 receptor (a generous gift from Prof. W. E. Paul, Bethesda, Md.) [14]. From these cell lines, 2×10^3 cells were added per well. The CTLL-2 was added in 50 µl/well RPMI/10% AB. The CT.h4 S was added in 50 µl/well RPMI1640 containing 5% Hyclone FCS, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol. As a control for the response of the cell line, a three-fold dilution series of human recombinant IL-2 or IL-4 (kind gifts from Dr. E. Liehl, Novartis Research Institute, Vienna, Austria) was prepared, ranging from 100 U/ml to 0.005 U/ml in 50 µl. To these series, 2×10^3 cells of the respective cell lines were added. The plates were incubated for 24 h at 37 °C in 5% CO_2 and then pulsed with 0.5 µCi [H_3]thymidine per well for 18 h. Plates were harvested on glassfibre filters and counted in a 1205 Betaplateô scintillation counter (Wallac Oy, Turku, Finland).

Statistical analysis

The data recorded in the HTL assay were included in the analysis only if proliferation curves of the cell lines, responding to the dilution series of recombinant interleukins, were linear around a half-maximal proliferation at a concentration of approximately 1 U/ml. For the calculation of the frequencies of alloreactive HTL, background values were determined as the mean plus 3 SD of the [^{3}H]thymidine incorporation in the 24 wells with only stimulator cells. All wells exceeding this background value were considered positive for IL-2 or IL-4 production. When the dilution series contained more than five dilutions with only negative or only positive wells, the results were excluded from the analysis. The observed numbers of negative wells at each dilution were analysed using the jackknife version of the maximum likelihood method, as described by Strijbosch et al., which is a validity test for the single-hit Poisson model [31]. When the frequency estimate is given, the number of wells *expected* to be negative at each dilution can be calculated as described by Taswell [33]. This was done to demonstrate differences between the *observed* and the *expected* number of neg-

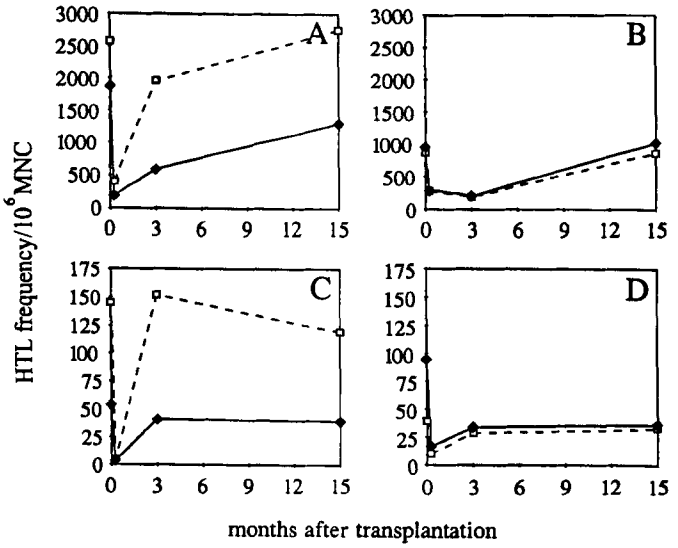

Fig. 1 A–D Follow-up of helper T-lymphocyte (HTL) frequencies after heart transplantation (HTX). Frequencies of IL-2 and IL-4-producing HTL were determined per 10^6 blood mononuclear cells (MNC), before and at three time points after HTX, towards donor (-◆-) and third party (-□-). In most patients, the frequencies dropped shortly after HTX, but returned to pre-HTX values later after HTX. Two representative examples show the follow-up for IL-2 and for IL-4: **A** IL-2, patient F; **B** IL-2, patient **C** C: IL-4, patient F; **D** IL-4 patient C (see Table 1)

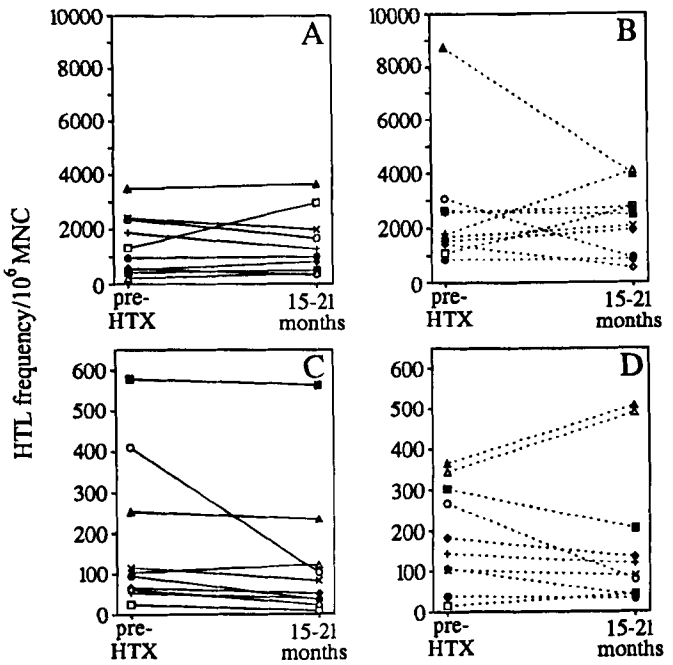

Fig. 2 A–D Frequencies of IL-2- and IL-4-producing HTL before and at 15–21 months after HTX. Frequencies were measured per 10^6 peripheral blood MNC of ten patients, towards donor and third-party spleen cells. **A** IL-2, donor; **B** IL-2, third party; **C** IL-4, donor; **D** IL-4 third party. No significant decrease or increase is observed after 15–21 months, compared to pre-HTX values. Patients from Table 1: A (■), B (□), C (●), D (○), E (×), F (+), G (◆), H (◇), I (▲), J (Δ)

ative wells. Frequencies are presented as number of responding cells per 10^6 blood MNC.

For comparison of data within groups of patients the Students *t*-test was used, the frequencies being normalized by ^{10}log transformation. Correlation analysis was performed using the Spearman rank correlation test.

Results

Longitudinal analysis of HTL frequencies

Frequencies of IL-2- and IL-4-producing HTL were analysed in peripheral blood MNC of ten HTX patients at four time points: before HTX, and then at 1–2 weeks, at 3–5 months, and at 15–21 months after HTX. Samples were chosen at time points without acute rejection, because cytokine production by activated T cells involved in the rejection process could interfere with the measurement of cytokines produced by T cells involved in the induction of nonresponsiveness.

In all patients, IL-2 frequencies were about 10-fold the IL-4 frequencies. For both IL-2 and IL-4, HTL frequencies in most patients dropped immediately after HTX to a mean of 50–60% of the pre-HTX values. This phenomenon occurred both towards donor and towards third-party spleen cells, and correlated with the drop in the percentage of T cells in the blood MNC, which was observed after start of the immunosuppression (data not shown). In most patients, frequencies were increased at 3–5 months after HTX, and had returned to values comparable to pre-HTX frequencies at 15–21 months after HTX. Representative examples are shown in Fig. 1.

In two patients, the HTL frequencies for IL-2 remained at a constant level during the follow-up. In one patient the frequency increased at 1 week after HTX, but returned to the pre-HTX level later after HTX. For IL-4 the same phenomenon occurred, but not always in the same patient as for IL-2. In three patients, after the initial drop the frequencies remained low until 15–21 months after HTX. When pre-HTX frequencies were compared with frequencies long after HTX significant changes were observed neither for IL-2 nor for IL-4, as shown in Fig. 2.

Comparison of completely mismatched versus partly matched patients

Patients were divided into two groups. Five patients (A, B, C, D, E, Table 1) were completely mismatched to the donor for at least HLA-DR, and with one exception,

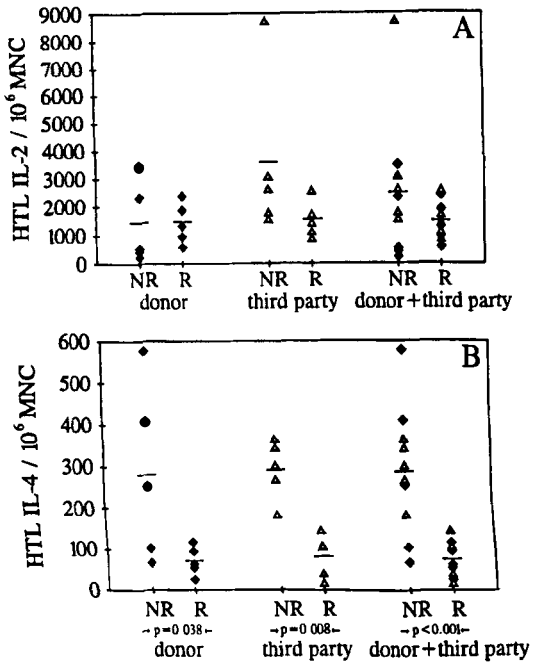

Fig. 3 A, B IL-2- and IL-4-producing HTL frequencies in rejectors and nonrejectors. Frequencies of **A** IL-2- and **B** IL-4-producing HTL per 10^6 blood MNC before HTX were compared in rejectors (*R*) and nonrejectors (*NR*), towards donor (◆), third party (Δ) and both. For IL-2, no significant differences are observed between rejectors (with treated rejection episodes after HTX) and nonrejectors (without treated rejection episodes). However, nonrejectors had a significantly higher frequency of IL-4-producing HTL than rejectors. Students *t*-test *P*-values were calculated after ^{10}log transformation of the frequencies

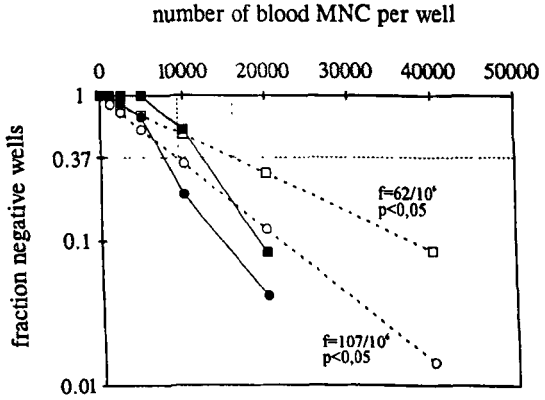

Fig. 4 Representative examples of IL-4-producing HTL frequencies that do not fit with single hit kinetics. The data were are obtained from patient H before transplantation, against donor (*squares*) and third party (*circles*). For each given frequency estimate, observed (*closed symbols*) and expected (*open symbols*) fractions of negative wells are depicted. The frequency is calculated from the number of MNC per well when the expected fraction of negative wells is 0.37

also for HLA-DQ. The other five patients (F, G, H, I, J) were matched to the donor for at least one HLA-DR and one HLA-DQ allele. The rejection scores were not significantly different between the two groups: 4.2 ± 4.5 versus 5.8 ± 4.2 ($P > 0.05$). Comparison of the pre-HTX frequencies, the percentage of the drop immediately after HTX, and the speed of recovery of the frequency longer after HTX, both for IL-2 and for IL-4, did not reveal any significant difference between the groups.

Comparison of rejectors versus nonrejectors

Patients were again divided into two groups, but this time based on their rejection score. Five patients (B, C, E, F, H, Table 1) suffered from several rejection episodes, including at least one episode that required additional treatment (grade 3A or worse, rejectors). The other five patients (A, D, G, I, J) did not suffer from any rejection episode, or had rejection episodes that did not require additional treatment (grade 2 or milder, nonrejectors). When pre-HTX frequencies of IL-2-producing T cells were compared, no difference was observed between the groups (Fig. 3A). However, pre-HTX frequencies of IL-4-producing T cells did differ between the groups: in the group of nonrejectors the frequencies were higher than in the group of rejectors (Fig. 3B). When the frequencies were ^{10}log transformed and evaluated using Student's *t*-test, this difference was statistically significant ($P = 0.038$). The difference was not donor specific, but was also observed in the reactivity towards third-party spleen cells ($P = 0.008$). When donor and third-party values were combined for the evaluation, the difference was even more pronounced ($P = 0.0004$). After 15–21 months, when most of the frequencies had returned to pre-HTX levels, the frequencies towards donor and third-party cells were still higher in the group of nonrejectors than in the group of rejectors ($P = 0.0167$ towards donor, $P = 0.0145$ towards third party, $P = 0.0005$ towards donor plus third party, data not shown). The pre-HTX frequency estimates for IL-4 are summarized in Table 2. Most of the frequencies for IL-4, as indicated in Table 2, did not fit with single-hit kinetics, ($P < 0.05$). This is in contrast to the frequencies for IL-2 (data not shown). Figure 4 demonstrates representative examples of data for IL-4, which did not fit with single-hit kinetics and shows that, after an initial delay, the observed fraction of negative wells decreases faster than expected, with increasing numbers of MNC per well. This may indicate that perhaps an extra stimulus is required before IL-4 production can be initiated, resulting in a multiple-hit process.

Pre-HTX frequencies were not only compared between groups of patients; the individual frequencies were also evaluated in correlation with the individual rejection scores. The individual frequencies of IL-2-producing T cells reactive to donor cells did not correlate

Table 2 HTL frequencies for IL-4 in nonrejectors and rejectors before heart transplantation. Patients are coded as in Table 1. Nonrejectors did not suffer from rejection episodes requiring additional immunosuppression, whereas rejectors did. Frequencies are indicated per million blood mononuclear cells. The 95% confidence intervals *(CI)* and Chi-square values from the statistical analysis of the single-hit Poisson model are given

Patient	Donor			Third party		
	Frequency	95% CI	χ^2	Frequency	95% CI	χ^2
Nonrejectors						
A	579	428–731	9.8[a]	303	232–374	12.2[a]
D	410	308–512	6.9[a]	268	195–340	1.7
G	66	51–81	10.5	184	137–231	2.5
I	254	194–315	7.1	366	276–457	7.3[a]
J	103	79–126	14.9[a]	345	267–423	15.4[a]
Rejectors						
B	25	18–32	23.6[a]	16	11–20	18.5[a]
C	95	75–116	11.4[a]	39	31–47	25.7[a]
E	117	92–142	17.5[a]	104	82–125	16.6[a]
F	54	42–66	10.3	145	112–178	10.3[a]
H	62	50–75	22.1[a]	107	82–131	12.5[a]

[a] Frequency estimates are significant at the 5% level, indicating that the data do not fit with the single-hit Poisson model (see also Discussion section)

with the rejection scores ($R = 0.153$, $P = 0.673$); the same held for the combined frequencies of IL-2-producing T cells reacting to donor and third-party cells ($R = -0.210$, $P = 0.374$). When individual frequencies of IL-4-producing T cells from the ten patients reactive to donor cells were correlated with the rejection scores the correlation was almost significant ($R = -0.624$, $P = 0.054$). When frequencies of IL-4-producing T cells responding to donor and third-party cells were combined the correlation was significant ($R = -0.676$, $P = 0.0011$).

Discussion

We analysed the frequencies of HTL in peripheral blood of heart transplant recipients before HTX and the changes in the HTL frequencies at various time points after HTX. The frequencies of both IL-2- and IL-4-producing HTL were analysed by LDA.

After HTX, frequencies for both IL-2 and IL-4 dropped rapidly in most patients. This is due to the start of the strong immunosuppressive therapy, consisting of cyclosporin A (CsA), prednisone and azathioprine. This therapy causes a reduction of about 50% in the percentage of T cells in the total blood MNC (not shown). In patients treated with an induction therapy with OKT3 (anti-T cell antibodies), we observed an almost complete loss of T cells in the MNC, accompanied by a comparable loss in HTL frequencies, indicating that T cells are the main source of IL-2 and IL-4 in our assay (not shown). In addition to this, several of the drugs inhibit T cell function by suppressing production of such cytokines as IL-2 and IL-4 [20, 35]. The frequency of IL-2-producing HTL in cardiac allograft recipients has been studied previously [4]. In that study, HTL frequencies were corrected for the number of T cells per well. Despite this correction, HTL frequencies were still lower 1–3 months after HTX than before HTX, which was explained as a possible effect of deletion of alloreactive T cells in the development of graft tolerance. However, we observed that after 1–2 years frequencies had returned to pre-HTX values in most patients. At that time point, the number of T cells had also returned to normal values (not shown). Therefore, we believe that the reduced activity observed at 1–3 months is more probably due to the functional suppression of T cells by the immunosuppressive treatment. When the dose of immunosuppression, especially of prednisone, is reduced later after HTX, both T cell numbers and T cell function are less markedly suppressed, which may account for the return of the HTX frequencies to pre-HTX values after 1–2 years.

Frequencies of IL-2-producing HTL were much higher than frequencies of IL-4-producing HTL. IL-2 plays a central part in the immune response, and is required for the activation and proliferation of both $CD4^+$ and $CD8^+$ T cells. Primary activation of naive $CD4^+$ T cells results in the production of only IL-2. After further activation, IL-4 and IFNγ are also produced [12, 28]. T cells then differentiate further into HTL producing different cytokine profiles, which may result in different frequencies for IL-2 and IL-4. The order in the events after primary T cell activation, with IL-2 production preceding IL-4 production, may also explain why the data for IL-4 did not fit with single-hit kinetics. Production of IL-4 after T cell activation may require a

multiple-hit process, requiring an additional initiating factor or stimulus, such as the production of IL-2 itself. One could speculate that if that initiating factor or stimulus is the limiting step for IL-4 production, and if sufficient potential IL-4-producing HTL are present, more wells will become positive for IL-4 production when the required concentration is reached than would be expected if IL-4 were the only factor involved. In our view, however, this phenomenon does not detract from the significance of the differences observed between the different groups of patients.

We previously demonstrated that the CTL frequency long after HTX was reduced in HTX recipients, which could indicate the development of nonresponsiveness [13]. We now show that this reduction is not accompanied by a decrease in IL-2-producing HTL or by an increase of potentially "tolerizing" IL-4-producing HTL. In kidney allograft recipients, a reduction in CTL and HTL frequencies was observed in only few patients. For IL-2-producing HTL, a reduction in the frequency was observed in only 5 out of 19 patients. In 2 of these 5 patients, this coincided with a reduction of CTL frequencies. In 1 patient, only the CTL frequency decreased [38]. Although the number of patients with a decreased CTL frequency differs from our previous observations, the study confirms that a reduction in the frequency of CTL does not necessarily coincide with a change in the HTL frequency. The fact that, in our study, total HTL frequencies long after HTX are comparable to preHTX frequencies indicates that the developing nonresponsiveness to donor antigens is at least not due to a deletion of donor-reactive HTL, but may be the result of qualitative changes in the function or regulation of the HTL.

For heart transplantation, the occurrence of HLA-DR matches between donor and recipient can only be determined retrospectively. In large groups of patients, a match for HLA-DR has been shown to be beneficial for survival after transplantation [11]. However, in our ten patients no differences were observed in rejection scores or in frequencies for IL-2 and IL-4, either before or after HTX, between patients who had one or two HLA-DR matches and patients who had two HLA-DR mismatches. This can be due to reactivity towards mismatches in other MHC class II alleles or loci. Furthermore, $CD4^+$ T cell reactivity can also be induced towards MHC class I antigens via indirect antigen presentation [8]. Therefore, matching for MHC class II is not necessarily sufficient to prevent a $CD4^+$ T cell-mediated response. In addition, although $CD4^+$ T cells are generally accepted to be the main producers of cytokines, $CD8^+$ T cells can also contribute to their production [24]. Thus, mismatches in MHC class I recognized by $CD8^+$ T cells may also induce production of IL-2 and IL-4 in our assay, because we do not discriminate different T cell subsets.

T cells are not only involved in the induction of rejection, but also have an important role in the regulation of tolerance induction [3, 6, 23]. These differential effects may depend on the cytokines produced by the T cells. IL-2 and IFNγ may promote the rejection response, whereas IL-4, especially, may inhibit T cell reactivity towards the donor tissue [10, 19, 32]. We made an interesting observation upon splitting up the patients in rejectors and nonrejectors based on their rejection scores. Patients who experienced no or only mild rejection episodes requiring no additional treatment (grade 2 or milder) appeared to have a significantly higher frequency of IL-4-producing HTL before and long after HTX than did those who experienced more severe rejection episodes (grade 3A or worse). It could be speculated that IL-4 may be involved in suppressing T cell responses against donor tissues, thereby reducing the incidence of graft rejection. No such difference was observed for IL-2. This last observation fits with findings in kidney graft recipients, where CTL and IL-2-producing HTL frequencies were not predictive for graft survival [2].

The higher frequencies for IL-4 were observed towards both donor and third-party spleen cells, indicating that the phenomenon does not involve a donor-specific regulatory mechanism. It seems more likely that differences between individuals account for the observed differences in the frequencies, resulting in a natural polymorphism of cytokine expression. This polymorphism could have a genetic background. For several cytokines, genetic polymorphisms appear to reside in the promoter region of the gene. These polymorphisms influence the transcriptional activity of cytokine genes, thereby affecting the level of production of cytokines [21, 34]. For IL-10 and TNFα, these promoter polymorphisms were shown to be linked to the risk of early rejection episodes in human heart transplant patients [34]. Also for the IL-4 gene, several promoter polymorphisms have been described [21, 30]. What we measured in our study, however, is not a higher transcriptional activity of the IL-4 gene at the individual cell level, but a higher overall frequency of T cells producing IL-4. Although the basis of this phenomenon may be different from the gene polymorphisms mentioned, a higher frequency of HTL producing IL-4 in an allogeneic response could still lead to a better prognosis in heart transplantation. This beneficial effect could be ascribed to the role that IL-4 can play in the down-regulation of the production of IL-2 and IFNγ, which are believed to be involved in the rejection process [27]. This would then confirm the Th1/Th2 paradigm for transplantation. However, we previously demonstrated that both Th1 and Th2 cytokines are expressed inside the graft during rejection of cardiac allografts [36]. The same has been described for e.g. kidney graft rejection [9, 15]. Nevertheless, even if rejection in humans may involve both Th1 and Th2 cytokines, a high frequency of IL-4-pro-

ducing HTL in the periphery can still exert its function by modulating graft-reactive T cells and their expression of cytokines.

The data presented here may point to an important mechanism, involving regulation of IL-4 production. The observed association between high frequencies of IL-4-producing HTL and low incidences of rejection may be the result of a direct or indirect influence of IL-4 on the regulation of the induction of rejection episodes. These data have been obtained from a small group of patients. For a further analysis of the mechanisms responsible for the high frequency of IL-4-producing HTL in certain patients, this study obviously needs to be extended. A detailed evaluation of the expression and polymorphisms both of IL-4 and of other cytokine genes, will provide new insights into the role of the cytokine network in allograft rejection. This may eventually lead to novel prognostic tests for the outcome of organ transplantation in individual patients.

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