

Interleukin-2 in CD8+ T cells correlates with Banff score during organ rejection in liver transplant recipients

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Abstract The aim of this study is to compare the histological grading of acute organ rejection according to the Banff score with intracellular interleukin-2 (IL-2) concentrations in cytotoxic CD8+ T cells from peripheral blood samples. 66 recipients after liver transplantation and 20 healthy controls were included into this study. Blood samples of liver transplant recipients were collected beside routine visits or, in case of suspected organ rejection, with additional liver biopsy. For cytometry, the blood cells were stained with CD3, CD8 and intracellular-IL-2. The percentage of cells with detectable intracellular IL-2 was significantly increased in patients with acute rejection ($n = 7$, $P < 0.001$, t Test) compared to recipients without rejection. The percentage of cells with detectable intracellular IL-2 (mean \pm SEM) was $7.6 \pm 0.9\%$ in rejection patients, $2.3 \pm 0.22\%$ in stable liver transplant recipients, and $14 \pm 2.99\%$ in healthy controls. Intracellular IL-2 correlates to the Banff score in rejection patients (Spearman's-rho = 0.81, $P < 0.05$). This cytometric method

shows a good sensitivity (71%) with a cut-off based on a high specificity of 95% for histological proven organ rejection in our study cohort. Measurement of intracellular IL-2 in cytotoxic CD8+ T-lymphocytes by flow cytometry correlates very well to the histological grading according to the Banff score and shows a good sensitivity and excellent specificity in acute organ rejection.

Keywords Liver transplantation · Rejection · Interleukin-2 · Flow cytometry · Banff score

Introduction

Immunosuppressive therapy following organ transplantation plays a key role for survival of both allograft and recipient. Cellular or acute rejection is characterized by activated cytotoxic T cells and is initiated by the presentation of donor HLA-antigens to host T cells within the graft which, via the secretion of interleukin (IL)-2, recruit activated T cells into the graft resulting in tissue damage. When compared to other types of solid-organ grafts, human liver transplants (LT) seem to be immunologically privileged, as suggested by several clinical observations. Thus, liver transplantation remains a highly effective treatment for end-stage liver disease. Despite calcineurin inhibitors (CNI), acute cellular rejection occurs in transplant recipients. Typical rejection management involves optimization of baseline immunosuppression and methylprednisolone boluses. However, 28–35% of patients do not respond to high-dose steroid therapy and require further treatment [1–5].

IL-2 has been shown to promote proliferation of activated T cells by engaging the IL-2 receptor, thereby performing a crucial role in the mediation of allograft

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rejection. The IL-2 receptor is composed of three primary subunits, known as CD25, CD122, and CD132. Basiliximab and daclizumab are high-affinity IL-2 receptor monoclonal antibodies that act by binding to these receptors, stopping normal T-cell proliferation and thereby preventing progression of acute cellular rejection. Immunodynamic studies have shown that daclizumab blocks and down regulates IL-2 receptors via suppression of CD25-positive cells for several weeks [6–8].

Cyclosporine and tacrolimus are part of standard immune suppression. It is well described that anti-calceinurins diminish IL-2 production in cytotoxic T cells. The calcineurin activity and IL-2 expression correlate with anti-calceinurin blood levels, but the prediction of events like acute rejection has not yet been well studied, and makes it difficult to determine them without liver biopsy [9–12].

Therefore, the aim of this study is to establish a diagnostic tool for detecting early stages of rejection in LT recipients by analyzing the intracellular IL-2 in cytotoxic CD8+ T cells. Furthermore, we compare the noninvasive detection of intracellular IL-2 in cytotoxic CD8+ T cells from blood samples to the histological grading of acute organ rejection according to the Banff score.

Materials and methods

86 individuals were included into this prospective study from March 2005 to April 2007. Besides samples from 66 LT recipients, 20 samples from healthy volunteers were collected via our blood donor bank. The local ethics committee approved the protocol especially with regard to taking additional blood samples. All patients gave their written informed consent. Only LT recipients who received CNI as mainstay immunosuppression were included in this study.

Blood samples of recipients were collected in addition to routine testing (e.g., blood count, clinical chemistry, CNI CO levels, and coagulation tests) or in case of a suspected organ rejection indicated by elevated liver enzymes. In this case, immediate liver biopsy in Menghini technic was performed within 36 h including confirmation of rejection from pathology and Banff score grading [13].

White blood cells for flow cytometric analysis were separated using a ficoll gradient (Sigma, Munich, Germany) and then processed with cytofix/cytoperm and golgi stop (Beckton Dickinson, Heidelberg, Germany). After 18 h, the cells were stained with fluorochromes for CD3+ (FITC), CD8+ (PE-Cy5) and intracellular IL-2 (PE) (Beckton Dickinson, Heidelberg, Germany).

The cells were double-gated (CD3+ and CD8+) for the analysis of cellular percentage for intracellular IL-2 with a

flow cytometer (FACScalibur, Beckton Dickinson, Heidelberg, Germany).

Statistical analysis comprised *t* test, Kolmogorov-Smirnov-test to check the assumptions of the *t* test, Spearman rank correlation test and Fisher's exact test. Box-Whisker-plots were used to illustrate the different frequencies of detecting intracellular IL-2 between the groups. All tests were two-tailed and *P*-values equal to or lower than 0.05 were considered significant. A one-sided 95% reference region for the percentage of cells with detectable IL-2 in stable LT recipients was used as a diagnostic test for a possible rejection in all LT recipients. All tests were calculated on an Apple Macintosh (Apple, Cupertino, CA, USA) with SPSS (SPSS Inc., 11.0.4, Chicago, IL, USA) for Apple MAC OSX.

Results

Among the 66 LT recipients in this study, we have detected 7 (10%) patients with acute rejection confirmed by liver biopsy. The demographic data are listed in Table 1.

The percentage of CD8+ cells with detectable intracellular IL-2 (mean \pm SEM) was $7.6 \pm 0.9\%$ in LT recipients with a histological confirmed rejection ($n = 7$), $2.3 \pm 0.2\%$ in stable LT recipients ($n = 59$), and $14 \pm 3.0\%$ in healthy volunteers ($n = 20$) (Fig. 1).

In LT recipients with a histological confirmed rejection, significantly higher percentages of CD8+ cells with detectable intracellular IL-2 were observed compared to stable LT recipients ($P = 0.003$, *t* test for log percentages of cells, Fig. 2).

Furthermore, we found a good correlation for the percentage of cells with detectable intracellular IL-2 and the

Table 1 Demographic data and indication for liver transplant recipients

	Stable TX	Rejection
Age (years)	54 \pm 11	54 \pm 14
Gender: Male (<i>n</i>)	41	5
Gender: Female (<i>n</i>)	18	2
Total (<i>n</i>)	59	7
Years after transplantation	7.13 \pm 3.5	7.09 \pm 3.6
Alcohol (%)	20	57
Hepatitis B (%)	32	0
Hepatitis C (%)	22	14
Autoimmune hepatitis (%)	1.6	14
Primary sclerosing cholangitis (%)	3.3	14
Primary biliary cirrhosis (%)	6.7	0
Other (%)	13	0
Banff score \pm SD	NA	5.8 \pm 1.8

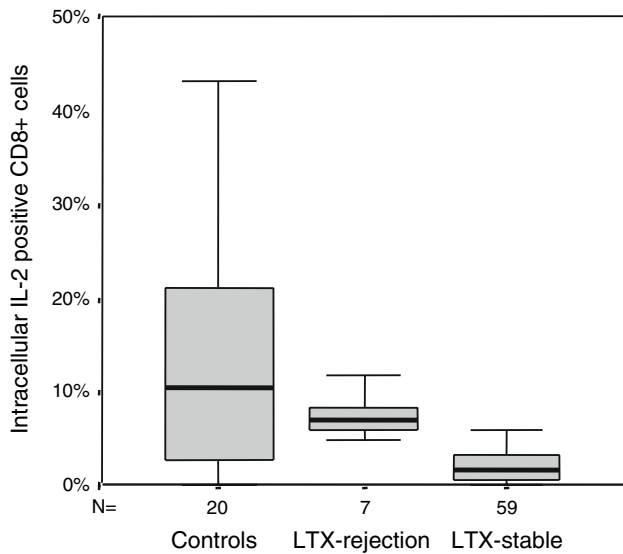


Fig. 1. Percentage of intracellular IL-2 positive cytotoxic CD8+ T-lymphocytes in the different populations

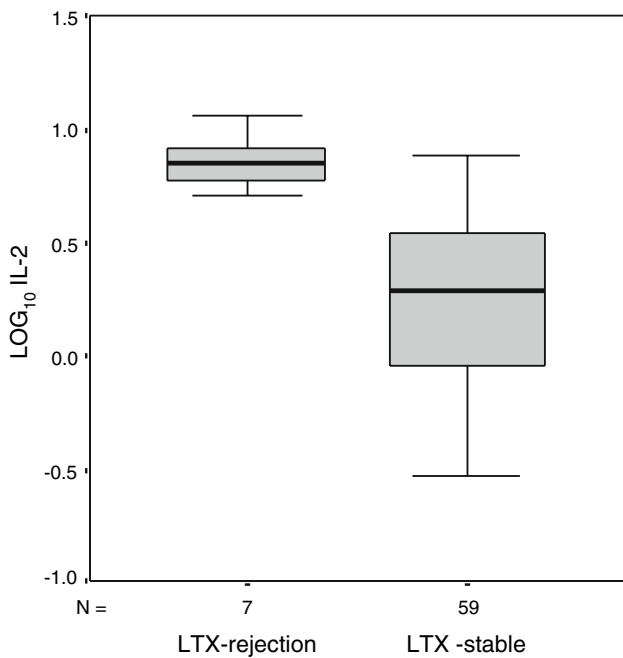


Fig. 2. Log percentage of CD8+ cells with detectable IL-2 in transplant recipients

Banff score (Spearman's rho = 0.81, *P* = 0.027, *n* = 7) in patients with acute rejection.

A one-sided 95% reference region for the percentage of cells with detectable intracellular IL-2 was determined with an upper bound of 6.58% in stable LT recipients. This corresponds to a high estimated specificity of 95% when used as a diagnostic test for rejection in all transplant patients and to a sensitivity of 71% in our study cohort (Table 2).

Table 2 Performance of the one-sided 95% reference region of the percentage of CD8+ T-lymphocytes with detectable IL-2 in stable liver transplant recipients as a diagnostic test for rejection in liver transplant recipients

Criteria for rejection	IL-2 > 6.58%
True/false positive	6/5
True/false negative	58/1
Sensitivity	71.4%
Positive predictive value	83.3%
Negative predictive value	96.7%

Discussion

Liver transplantation is widely accepted as an effective therapeutic procedure for a variety of irreversible acute and chronic liver diseases. The success of liver transplantation has increased steadily over the last two decades and several advances have been made since the first human liver transplantation.

The results of liver transplantation have improved due to advances in preoperative treatment, excellent surgical skills, better understanding of the course and prognosis of several liver diseases, improved immunosuppressive therapy and more effective aftercare.

In recent studies, the incidence of acute organ rejection after liver transplantation was reported to be 18–19% [14]. In our patients studied, we have detected 10% acute rejections, which were confirmed by liver biopsy with grading according to the Banff score. This lower percentage compared to other studies might be due to the relatively small sample size of our cohort.

The aim of our study was to evaluate the percentage of cytotoxic CD8+ T cells with detectable intracellular IL-2 in patients during acute organ rejection, compared to stable transplant recipients and healthy volunteers.

In patients with acute liver allograft rejection, the study of Boleslawski et al. [15] describes a percentage of intracellular IL-2 of about 17% in CD8+ T cells. These data obviously differ from our findings (7.6 ± 0.9%) and are almost like the mean percentage of intracellular IL-2 positive CD8+ cells in our healthy volunteers. We have detected a 3.2-fold higher number of CD8+ IL-2 positive cells in rejection patients compared to stable transplant recipients. Boleslawski et al. could show a 2.25-fold higher number of cells. The reasons for these differences are probably based on two facts: Boleslawski and coworkers gated CD8+ cells in their setting and after initial isolation the cells were also chemically stimulated in vitro. However, we double gated CD3+ and CD8+ cells before measuring the intracellular IL-2 in CD8+ cells without any stimulation. The histological diagnosis of allograft rejection is based on established histological classifications like

the Banff score [13]. Nevertheless, results might differ for scoring and grading and can show inter observer variation [16, 17]. Therefore, the implementation of a second diagnostic test may be used in combination with the histological diagnosis of acute allograft rejection.

Our study shows that intracellular IL-2 in cytotoxic CD8+ T cells correlate well to the Banff score in LT recipients with acute organ rejection; furthermore, only patients with CNI as mainstay immunosuppression were included into this study. Other substances like rapamycin or mycophenolate mofetil, which have a different way of action to achieve immunosuppression were excluded. Rapamycin or mycophenolate mofetil may have different effects on basal intracellular IL-2 compared to CNI in stable recipients. However, it remains speculative if during acute rejection the percentage of intracellular IL-2 positive cells is supposed to increase like in CNI based therapy protocols.

The role of IL-2 is crucial in acute organ rejection [18]. Our data suggest that different stages of immune activation (rejection vs. no rejection and immune suppression vs. no immune suppression) show different percentages of intracellular IL-2 positive cells. As mentioned before, all patients studied received CNI as mainstay therapy. Interestingly, there was no significant variability in calcineurin blood levels measured (data not shown). Therefore, we suggest that immunosuppressive therapy with calcineurin inhibiting substances does not influence the production of IL-2 in CD8+ cytotoxic T cells during organ rejection.

Our results suggest a good sensitivity and high specificity between acute rejection and stable transplant recipients. Due to the small proportion of acute rejection of only 10%, the positive predictive value is less supportive but the negative predictive value is again high.

Nevertheless, further studies with different immunosuppressive strategies are needed to evaluate this method as an additional way to detect rejection and as a challenge to the established histological approach.

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Conflict of interest statement None.

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