Cytokine gene polymorphisms in liver transplantation

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Cytokine genpolymorfismen en levertransplantatie

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To my parents

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Chapter 1

General introduction

Introduction

Liver transplantation

The first four liver transplantations were performed in 1963; all four recipients died within the first month after transplantation. Therefore, the program was stopped for more than three years. In 1968 Thomas E. Starzl reported the first three cases of liver transplantation with extended survival and opened the era of liver transplantation (1). However, rejection was one of the major barriers to successful liver transplantation, causing patient 1-year survival of approximately 30% (2). The introduction of cyclosporin in 1979 by Sir Roy Calne and colleagues (3), revolutionized the results of liver transplantation. Already in 1983, the National Institute of Health covered a consensus conference to review the efficacy of liver transplantation and concluded that it was no longer an experimental procedure, but rather an appropriate therapy for many patients with end-stage liver disease (4). Nowadays, there are over 200 liver transplant programs and between 7000 and 8000 patients a year receive a liver transplant worldwide with expected 1- and 5-year patient survival of greater than 85% and 70%, respectively (5). As a consequence of the improved short-term survival, due to refinement of surgical techniques and better post-operative management, long-term outcome after liver transplantation is becoming the main concern for clinicians dealing with liver transplant patients. Recurrence of original disease, e.g. hepatitis B and C infection, hepatocellular carcinoma, and primary biliary cirrhosis, chronic rejection, and biliary complications have a clear impact on long-term patient survival. However, immunosuppression-related complications as infections, cardiovascular disease, renal failure and de novo malignancy are the most important determinants of long-term patient-survival and quality-of-life (6). Therefore, safe reduction of immuno-suppressive drugs tailored to specific patient's needs, or in some cases complete withdrawal of immunosuppression is a main topic in transplant medicine of today.

Acute liver graft rejection

Acute cellular rejection is the most common type of rejection seen following liver transplantation. It occurs in up to 50% of patients within the early post-transplant period depending on the immunosuppressive protocol used (7). Acute cellular rejection, however, does not appear to adversely affect long-term liver allograft survival (8). The first episode of acute cellular rejection usually occurs between 4 and 14 days following transplantation and most episodes of acute rejection occur within the first month after transplantation. Acute rejection is typically manifested by fever, with decreased bile output, and elevation of serum transaminases, γ-glutamyl transpeptidase, alkaline phosphatase, and bilirubin. Although the diagnosis of rejection may seem evident based on the clinical picture, a number of conditions can mimic rejection and should be considered when liver function becomes abnormal. In

particular, differentiation between acute rejection and infectious complications is a clinical challenge during the early post-transplant period. Therefore, liver biopsy is needed to obtain characteristic histological evidence of acute rejection including mononuclear portal infiltration, bile duct inflammation or injury and portal or central vein endothelialitis (9).

Immunology of liver graft rejection

The alloreactive immune response after liver transplantation can be divided into three successive stages: first, the recognition of alloantigens by naive host T cells; second, the activation and the expansion of alloreactive T cells; and third, the effector phase (10).

Acute allograft rejection is initiated when donor tissue antigens (alloantigens) present on the cell surface of the transplanted liver are recognized as foreign by T cells of the recipient's immune system. The recognition of alloantigens by recipient T cells can occur in two ways. Donor antigen-presenting cells including dendritic cells, macrophages, monocytes and B cells, present 'non-self' major histocompatibility complex (MHC) class I or class II molecules to the T cell receptor (TCR) of recipient T cells in a local lymph node (direct allorecognition). The second mechanism of allorecognition is the uptake of 'non-self' proteins by the recipient's own antigen-presenting cells and their presentation by 'self' MHC molecules to recipient T cells (indirect allorecognition) (11).

Optimal T cell activation not only requires allorecognition by the interaction between TCRs and foreign allo-antigens, but also interactions between co-stimulatory molecules on APCs and their ligands on T cells (12). In the absence of co-stimulation, allorecognition leads to T-cell anergy or deletion (13), whereas a TCR-signal in conjunction with co-stimulation leads to cytokine gene activation by the induction of transcription factors within the T-cell (14). Further proliferation and differentiation into helper- and effector T cells is driven by the cytokine, interleukin-2 (IL-2) which is produced by the activated helper T cell itself. The central importance of IL-2 is well illustrated by the drugs that are commonly used to suppress transplant rejection. Cyclosporin A and tacrolimus inhibit IL-2 production by disrupting signaling through the T cell receptor, anti-IL-2 receptor monoclonal antibodies (such as basiliximab) prevent the binding of IL-2 to its receptor, whereas rapamycin (sirolimus) inhibits the IL-2-mediated signal transduction pathway (15).

During the effector phase, activated T cells express integrins for binding to adhesion-molecules on activated endothelium at the site of inflammation (16) and they are guided to the liver by a chemoattractant gradient of chemokines released by the liver (17, 18). Subsequently, the infiltrating T cells mediate a number of effector mechanisms such as T cell cytotoxicity, delayed-type hypersensitivity by macrophage activation, and alloantibody production by the activation of B cells (10, 19). These

mechanisms cause tissue damage with the clinical signs and symptoms of acute liver graft rejection.

Cytokines and the Th1-Th2 paradigm

Cytokines are low-molecular weight proteins or glycoproteins which act as soluble mediators and regulators of immune responses. The family of cytokines includes interleukins, interferons, lymphokines, growth factors and chemokines (20). Cytokines are produced by a wide range of cell types including parenchymal cells (e.g. endothelial cells, and hepatocytes) and immune cells (e.g. T cells, B cells, monocytes, macrophages, and NK cells). They act on target cells by binding to specific cytokine receptors, initiating signal transduction and second messenger pathways within the target cell (21). This can result in gene activation, leading to mitotic division, growth and differentiation, migration, or apoptosis. Cytokines are active in low concentrations and their activity is local on the cell of origin (autocrine) or on nearby cells (paracrine). Furthermore, they can also act at a distance (endocrine), when they are produced in higher concentrations and released into the circulation. Cytokines act in a highly complex network in which they induce or repress their own synthesis as well as that of other cytokines. In addition, most cytokines are able to act on multiple target cells (pleiotropy) with considerable overlap between the function of individual cytokines (redundancy) (22).

The alloresponse is primarily orchestrated by a range of T cells with diverse functions. Cytotoxic T cells ($CD8^+$) are involved in cytotoxicity, while T helper cells ($CD4^+$) direct the differentiation and effector functions of other cells through the production of cytokines. T helper cells have been subdivided into different functional Th subsets on the basis of their cytokine production. Th1 cells promote cell-mediated effector respones and characteristically produce IL-2 and IFN-γ. Th2 cells regulate B cell mediated humoral responses, inhibit Th1 responses (21), and typically produce IL-4, IL-10 and IL-13. In humans however, the distinction between Th1 and Th2 cells is not very well defined, and a subset of Th0 cells, which produce cytokines typical of both Th1- and Th2 profiles can be identified (24). Furthermore, regulatory T cells with unique cytokine profiles distinct from that of Th0, Th1 and Th2 have recently been defined. Th3 cells produce TGF-β with variable amounts of IL-10, and T regulatory (Tr) type 1 cells produce IL-10 with or without TGF-β. Functional studies have shown that these Th3 and Tr1 cells have the ability to prevent the development of Th1-mediated immune responses by the release of immuno-suppressive cytokines including IL-10 and TGF-β (25, 26).

Gene polymorphisms

A genetic polymorphism is defined as the occurrence of two (or more) alternative alleles in a population at such a frequency that the rarest could not be maintained by

mutation alone (27). In practice, a genetic locus is considered polymorphic if the rare allele has a frequency of at least 1%. Single nucleotide polymorphisms (SNPs) are by far the commonest form of polymorphism (>97%) (28), and occur on average every 500-1000 base-pairs throughout the human genome (29, 30). Other types of gene polymorphism include insertions or deletions of one or more bases and microsatellite repeats (28).

There are about 30 to 40 thousand protein-coding genes (31) and of the estimated 1.42 million SNPs in the human genome an estimated 60.000 SNPs fall within these coding regions (exons) (32). SNPs within coding regions can result in loss or change of function in the expressed protein as a result of change in protein structure, whereas SNPs in non-coding sequences of the genome can influence gene expression if they occur in regulatory regions. For example, SNPs in the promoter region may alter the binding of transcription factors (33) and SNPs in the 3' untranslated region may affect stability and translation of mRNA (34). Polymorphisms affecting protein function (coding region SNPs) or gene expression (regulatory region SNPs) are termed 'functional' polymorphisms, whereas 'anonymous' polymorphisms do not have biological consequences. Another class of polymorphisms are those in linkage disequilibrium with other truly functional polymorphisms or with loci or haplotypes associated with disease. These polymorphisms then act as markers of human disease (35).

Cytokine genotyping

Cytokines play a pivotal role in mediating allograft rejection, and therefore functional polymorphisms in cytokine genes might be useful to predict immune responsiveness of individual liver transplant recipients. Most of the reported polymorphisms in cytokine genes occur within known or putative regulatory regions (36) and some have been shown to influence cytokine gene expression. For example, the single nucleotide polymorphism at position –308 (G/A) in the promoter of the TNF-α gene has been associated with a six-fold increase in TNF-a transcription and increased *in vitro* TNF-α protein production (37, 38), whereas the G to A substitution at position –1082 in the IL-10 promoter was associated with decreased IL-10 protein production *in vitro* (39). Although, these associations could not always be confirmed by other studies (40-42), it has become accepted to denote cytokine gene polymorphisms by their presumed association with high or low *in vitro* cytokine production. Since the relationship between polymorphisms and cytokine production is not very well established, the research in this thesis does not focus on cytokine gene polymorphisms in relation to liver graft rejection alone, but also addresses the genetic basis for interindividual differences in cytokine production profiles and its influence on acute rejection after liver transplantation.

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Chapter 2

Aim and outline of the thesis

Aim of the thesis

The aim of this thesis is to identify polymorphisms in cytokine genes as factors involved in the regulation of cytokine production and the pathogenesis of acute rejection after liver transplantation. This may not only lead to better understanding of the mechanism of rejection, but may also result in the construction of a genetic profile that gives clinicians guidance on the adjustment of immunosuppressive therapy to the individual needs of liver transplant patients.

Outline of the thesis

In this thesis we present in four parts the relationship between cytokine gene polymorphisms, cytokine production and the severity of the inflammatory response in the context of liver transplantation. In ***part one*** the importance of cytokines early after liver transplantation is illustrated by the *in vivo* measurement of cytokine patterns during episodes of rejection and infection. Cytokines in bile may reflect the immunologic activity within the liver more closely than those in circulation. Therefore, we study whether early recognition of acute liver graft rejection and differentiation from infectious complications can be improved by combining cytokine patterns in serum and bile *(chapter 3)*. In ***part two*** we describe differences between individuals in the capacity to produce cytokines after mitogenic stimulation of peripheral blood mononuclear cells isolated from healthy controls and liver transplant patients. We investigated whether these interindividual differences in cytokine production profiles are related to the occurrence of acute liver graft rejection *(chapter 4)*. Since differential levels of cytokine production may be attributed to polymorphisms in regulatory regions of cytokine genes, we analyzed the relationship between cytokine gene polymorphisms and *in vitro* cytokine responses, both in liver transplant recipients and in healthy individuals. Also, we review the literature for the association between polymorphisms and *in vitro* cytokine production in various patient groups and healthy individuals *(chapter 5)*. In ***part three*** we present an animal model of liver transplantation. Liver allografts can be spontaneously accepted in fully mismatched donor recipient combinations of inbred rat strains, whereas in some MHC-identical combinations liver allografts reject in a short-term manner. Strain-specific *in vitro* cytokine production profiles may influence the severity of the allogeneic inflammatory response after liver allografting. Therefore, we assessed whether strain-specific *in vitro* cytokine production profiles can predict survival after liver transplantation in the rat *(chapter 6)*. To investigate whether differences in cytokine responses among inbred rat strains are genetically determined, we studied the relationship between polymorphism and *in vitro* expression of the TNF-α gene in different inbred rat strains *(chapter 7)*. In ***part four*** we analyzed the association of human liver allograft rejection with gene polymorphisms in cytokines throughout the whole cytokine

network *(chapter 8)*. Finally, we assessed the relevance of some cytokine gene polymorphisms with respect to acute rejection in a meta-analysis of seven comparable studies *(chapter 9)*.

Part I

In vivo cytokine patterns in liver transplant patients

Warlé MC, Metselaar HJ, Hop WCJ, Gyssens IC, Kap M, De Rave S, Kwekkeboom J, Zondervan PE, IJzermans JNM, Tilanus HW, Bouma GJ.
Early differentiation between rejection and infection in liver transplant patients by serum and biliary cytokine patterns.
Transplantation 2003; 75: 146-151.

Part I

Chapter 3

Early differentiation between rejection and infection in liver transplant patients by serum and biliary cytokine patterns

Differentiation between acute liver graft rejection and infection remains a clinical challenge during the early posttransplantation period. Although cytokines play a pivotal role in mediating allograft rejection, previous studies demonstrate that most cytokines are not specific for liver graft rejection or infections. However, other studies suggest that adhesion molecules and cytokines in bile reflect the immunologic activity within the liver more closely. Therefore, we postulated that by combining cytokine patterns in serum and bile, early recognition of acute liver graft rejection and differentiation from infectious complications can be improved. We performed a prospective study in 45 patients who were monitored daily for clinical events and cytokine patterns in serum and bile during the first month after liver transplantation. Soluble intercellular adhesion molecule-1 (sICAM-1) in serum and interleukin-8 in bile were specifically increased at the onset of acute rejection ($P<0.001$), whereas serum soluble tumor necrosis factor-receptor II was also significantly increased in patients with infectious complications and serum interleukin-6 only in patients with rejection during infection. In 68% of patients with increased sICAM-1, acute rejection was diagnosed within 10 days, whereas rejection occurred in only 26% of patients with low serum levels of sICAM-1. In patients with increased sICAM-1, the relative risk for rejection was 4.8 ($P=0.009$). Cytokine patterns in bile do not provide rejection markers with higher specificity compared with serum cytokines. Daily monitoring of sICAM-1 in serum could identify patients at risk for rejection; therefore, acute liver graft rejection may be recognized earlier in those patients.

Introduction

After liver transplantation, clinical signs and symptoms of acute rejection and infectious complications are similar. Acute liver graft rejection usually occurs within the first month after transplantation (1). During this early posttransplant period, most patients also experience one or more episodes of infection, which are the same nosocomial infections occurring in surgical patients who are not in a state of immunosuppression (2, 3). Deteriorating liver function can indicate rejection but is also seen in patients with organ/space surgical site infections (SSI), including bacterial peritonitis and cholangitis. Therefore, early recognition of acute rejection and differentiation from infectious complications remains a clinical challenge after liver transplantation. Previous studies have been performed to assess whether measurement of cytokines (4-15) and adhesion molecules (16-23) in serum or bile could differentiate between liver graft rejection and infection. These studies showed that increased levels of serum cytokines were not specific for any complication. However, one report showed that soluble interleukin-2 receptor (sIL-2R), soluble tumor necrosis factor-receptor II (sTNF-RII), and interleukin (IL)-10 were elevated before and during rejection, whereas an increase in IL-8, neopterin, and sTNF-RII was indicative of severe infection (6, 7). As local secretion of cytokines at the site of the inflamed portal tracts may provide specific markers of acute liver graft rejection, previous reports assessed the use of biliary sIL-2R (4), IL-6 (14), and soluble intercellular adhesion molecule-1 (sICAM-1) (17) in monitoring liver transplant patients. These studies showed that biliary sICAM-1 and sIL-2R levels were increased during liver graft rejection compared with patients with other (infectious) complications and stable graft function (4, 17). Biliary IL-6 was increased not only in patients with acute rejection but also in patients during episodes of cholangitis (14).

In this study, we hypothesized that by combining cytokine patterns in serum and bile, we could improve early differentiation between acute liver graft rejection and infectious complications. For this purpose, we performed a prospective study in which serum and biliary levels of sICAM-1, sIL-2R, sTNF-RII, IL-6, IL-8, and IL-10 were measured daily during the month after liver transplantation. Furthermore, we scored all episodes of rejection and infection according to Banff criteria and Centers for Disease Control and Prevention (CDC) definitions, respectively, of nosocomial infections and SSIs.

Materials and methods

Patients

Between June 1998 and April 2000, a total of 61 orthotopic liver transplantations were performed in 56 patients. Forty-five patients were consecutively enrolled in this study, whereas 11 patients were excluded (six patients without external biliary

drainage by T tube, three patients with short follow-up as a result of early intraoperative or postoperative death, one patient without selective bowel decontamination [SBD], and one patient for whom we did not obtain informed consent). Median age was 47 years and ranged from 22 to 67 years. Indications for liver transplantation included hepatitis B virus- and hepatitis C virus-related cirrhosis in 12 patients, primary sclerosing cholangitis or primary biliary cirrhosis in nine, acute hepatic failure in eight, cryptogenic cirrhosis in five, alcoholic cirrhosis in five, Wilson's disease in three, and other diseases in three. Three patients underwent retransplantation for primary nonfunction (n=2) and hepatic artery thrombosis (n=1). The study was approved by the ethics committee of the University Hospital Rotterdam, and informed consent was obtained before participation in the study.

Immunosuppression and antimicrobial prophylaxis

Immunosuppression was started as triple therapy (n=18), including prednisone, cyclosporine A, and azathioprine, or as double therapy (n=27), including prednisone with cyclosporine or tacrolimus (Prograf, Fujisawa, Deerfield, IL). Induction therapy with IL-2 receptor antagonist basiliximab (Simulect, Novartis, East Hanover, NJ) was given to 23 patients within 6 hr after surgery and at postoperative day 4. For treatment of acute rejection, patients received intravenous methylprednisolone at a dosage of 1,000 mg per day for 3 days. All patients included in this study received SBD (intravenous cefotaxime 1 g four times per day until cultures [throat and rectum] were negative for gram-negative bacteria, amphotericin B suspension 500 mg four times per day, and colistin/norfloxacin capsule 200/50 mg four times per day for 30 days posttrans-plantation).

Acute rejection and infectious complications

Acute liver graft rejection was defined as an episode of clinical signs (malaise, fever, jaundice, decreased bile volume, and decreased bilirubin level in bile), deteriorating liver function (elevated serum bilirubin, aspartate aminotransferase, alanine aminotransferase, γ-gamma-glutamyl transpeptidase, and alkaline phosphatase), and histology showing characteristic features of acute rejection according to the Banff criteria (24). The first day of treatment with a course of high-dose methylprednisolone was defined as the onset of acute rejection. All infectious complications were classified according to the CDC definitions of nosocomial and SSIs (25, 26). With respect to infectious complications, diagnosis according to CDC criteria and day of onset was determined by an infectious disease physician (I. G.) and hepatologist (H. M.) who evaluated clinical and laboratory data, including C-reactive protein, in a blinded fashion with regard to serum and biliary cytokine patterns.

Measurement of cytokines in serum and bile

Serum and T-tube bile samples were collected daily, early in the morning, for 1 month after liver transplantation. After centrifugation, serum and bile samples were stored at -80°C until assayed. Commercially available enzyme-linked immunosorbent assays with 96-well microtiter plates were used for measurement of sICAM-1, sTNF-RII, and IL-10 (Quantikine Immunoassay, R&D Systems Europe, Abingdon, UK). Soluble IL-2R, IL-6, and IL-8 were measured by means of chemiluminescent enzyme immunoassays for use with the IMMULITE automated analyser (Diagnostic Products Corporation, Los Angeles, CA).

Statistical analysis

For the comparison of groups, cytokine measurements at days after the first episode of rejection or infection were excluded to eliminate interference by previous episodes. Repeated measurements analysis of variance (ANOVA) was used to evaluate whether the use of double versus triple therapy and basiliximab as induction therapy affected average serum and biliary cytokine levels during the period of monitoring. ANOVA was also used to evaluate differences in average cytokine levels in patients with abdominal infections (n=11) versus other infections (n=7) and patients with rejection only (n=11) versus rejection during infection (n=4). The PROC MIXED program from the SAS-package (version 6.12) (SAS Institute, Cary, NC) was used for ANOVA. Cox-regression analyses with time-dependent variables were performed to evaluate whether serum and biliary cytokines were related to rejection. To assess the clinical relevance of serum and biliary cytokines as early rejection markers, cutoff levels were defined. For statistical analyses, all cytokine data were transformed logarithmically to reduce skewness of distributions. Results were expressed as means ± standard error of the mean.

Results

Postoperative monitoring

Within the first postoperative month, 15 of 45 patients (33%) were treated with a course of high-dose methylprednisolone for one or more episodes of biopsy-proven acute rejection. In 11 cases acute rejection was the first postoperative event, whereas in 4 cases acute rejection was diagnosed during an episode of infection. In patients who did not experience acute rejection, 11 (24%) experienced an uneventful postoperative course, whereas 18 patients (40%) experienced infectious complications. One patient experienced a fever of unknown origin. Most frequent infections were organ space / SSIs, including bacterial peritonitis (n=8) and cholangitis (n=5). Other infections were pneumonia (n=5), superficial incisional SSI, sinusitis, urinary tract infection, and Clostridium difficile colitis. Probably as a result of the administration of antibiotics as SBD, most infectious complications were caused by gram-positive

pathogens (Table 1). There were seven patients with gram-negative infections; four occurred after discontinuation of SBD. In three patients, gram-negative pneumonia occurred early, during administration of cefotaxime as part of SBD. There were no systemic viral infections during the first postoperative month. Two patients experienced a limited herpes labialis infection.

Table 1. Postoperative course of 45 consecutive liver transplant patients

		No patients
Acute rejection		
Rejection only		11
Rejection during infection		
Organ space/SSI	Coagulase negative staphylococci	2
Pneumonia	*Stenotrophomonas maltophilia*	1
	Pseudomonas aeruginosa and *Stenotrophomonas maltophilia*	1
Infectious complications[a]		
Organ space SSI	Coagulase negative staphylococci	4
	Staphylococcus aureus	1
	Enteococcus faecalis	1
	Enterococcus faecium	1
	Bacillus cereus and coagulase negative staphylococci	1
	Enterobacter cloacae	1
	Klebsiella pneumoniae	1
	Culture negative	1
Superficial incisional SSI	No culture	1
Pneumonia	*Pseudomonas aeruginosa*[b]	1
	Acinetobacter baumannii and *Stenotrophomonas maltophilia*	1
	Culture negative	
	Pseudomonas species	1
Sinusitis	*Enterococcus faecalis*	1
Urinary tract infection	*Clostridium difficile*	1
Colitis		1
Fever of unknown origin		1
Uneventful		11

[a] When patients had more than one infectious episode, patients were classified according to their first episode.
[b] Lethal infection.
SSI, surgical site infection

Effect of immunosuppression on cytokine levels

Of forty-five patients included in this study, 23 received basiliximab (IL-2 receptor antagonist) as induction therapy administered within 6 hr after transplantation and at postoperative day 4. Basiliximab reduced the incidence of acute rejection from 41% to 26 % (not significant). Administration of basiliximab at days 0 and 4 decreased average levels of sIL-2R (P=0.001) and IL-10 (P=0.007) in serum, whereas biliary

levels of sIL-2R were not significantly affected. ANOVA showed that serum and biliary levels of sICAM-1, sTNF-RII, IL-6, and IL-8 were not significantly affected by the administration of basiliximab (data not shown). Because the groups with (n=22) and without (n=23) basiliximab separately were too small to allow a meaningful statistical analysis, the relation with clinical events was not evaluated for sIL-2R and IL-10.

Furthermore, ANOVA showed that average levels of sICAM-1, sTNF-RII, sIL-2R, IL-8, and IL-10 were not significantly different between patients with double (cyclosporine or tacrolimus with prednisone; n=27) versus triple therapy (cyclosporine, prednisone, and azathioprine; n=18) as initial immunosuppression, whereas serum levels of IL-6 were decreased in patients who received triple therapy (P=0.005).

Cytokine patterns in patients with organ space/surgical site infections versus other infections

Before diagnosis, patients with organ space/SSIs demonstrated significantly higher average serum levels of sTNF-RII compared with uneventful patients (P=0.033), whereas serum sTNF-RII was not increased in patients with other infections (P=0.58). The increase of sTNF-RII levels before diagnosis of rejection did not reach statistical significance (Figure 1A; P=0.095). Average levels of serum sICAM-1, IL-6, IL-8, and biliary cytokines were not significantly increased in patients with organ space/SSIs and other infections.

Cytokine patterns in patients with rejection during infection versus rejection only

Before diagnosis, patients with rejection during infection (n=4) demonstrated significantly higher average serum levels of IL-6 (P<0.001) compared with patients with rejection only (Figure 1B). Average levels of serum sTNF-RII, sICAM-1, IL-8, and biliary cytokines were not increased in patients with rejection during infection compared with rejection only.

Cytokine patterns in relation to acute liver graft rejection

Cox-regression analysis showed that at the onset of rejection serum, sICAM-1 (mean 1,401±157 ng/mL, P<0.001) and biliary IL-8 (11,623±4,255 pg/mL, P<0.001) levels were significantly increased compared with patients with an uneventful course and infectious complications (Figure 1C and D), whereas other serum and biliary cytokines were not significantly increased at the onset of rejection.

In uneventful patients, mean levels of serum sICAM-1 and biliary IL-8 reached a maximum at postoperative day 3 (893 ng/mL and 17,823 pg/mL, respectively), whereas minimum levels were reached at approximately postoperative day 16 (529 ng/mL and 1,036 pg/mL, respectively). To define an upper limit of normal for

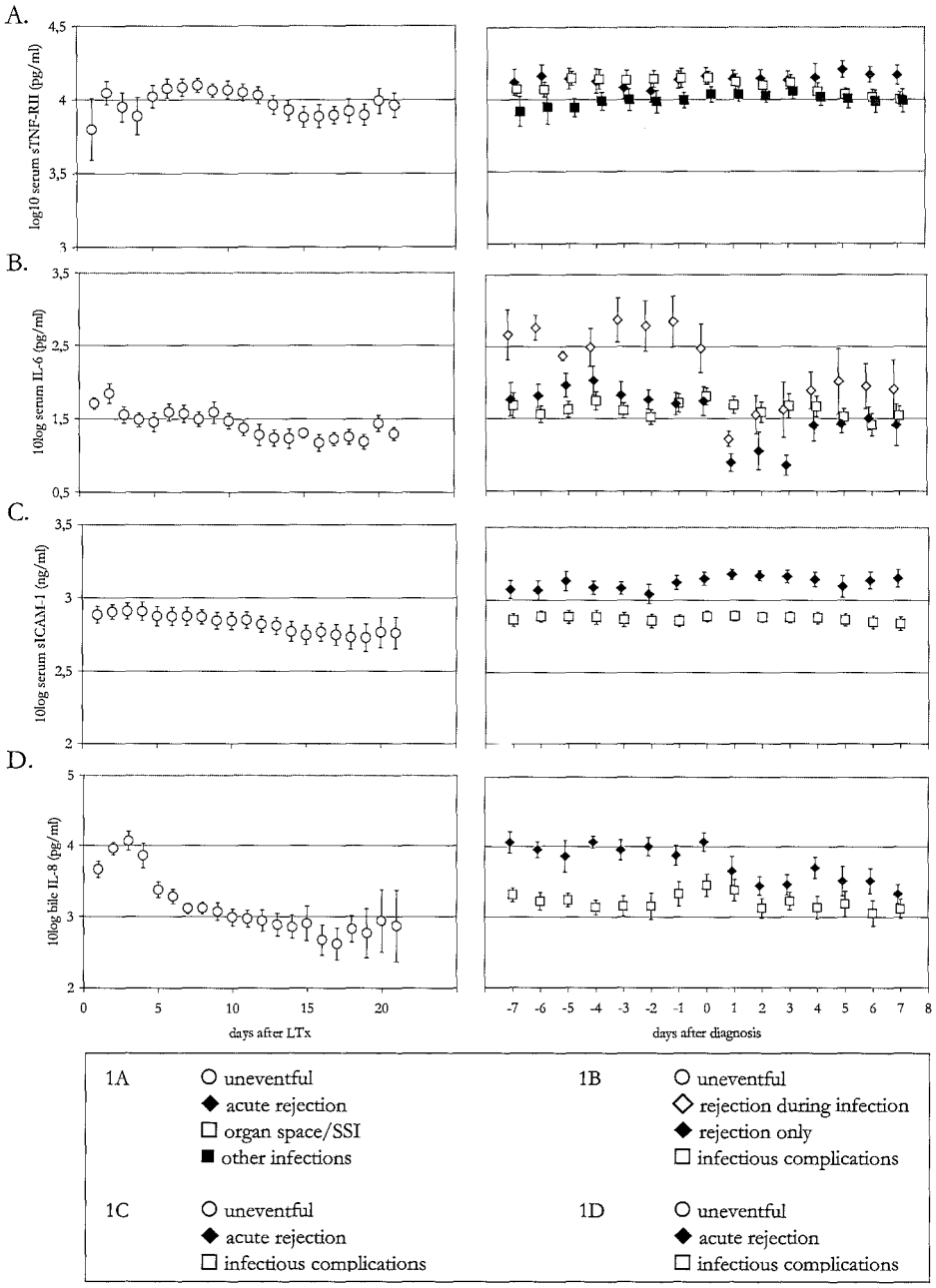

Figure 1. Serum soluble tumor necrosis factor-receptor II (sTNF-RII) (A), interleukin (IL)-6 (B), soluble intercellular adhesion molecule-1 (sICAM-1) (C), and biliary IL-8 (D) in liver transplant patients with an uneventful course (*left*) and patients with acute rejection or infectious complications (*right*).

serum sICAM-1 and biliary IL-8, the mean of uneventful patients plus two standard deviations was used as the cutoff at each postoperative day. In 52% of the patients who exceeded the biliary IL-8 cutoff level, acute rejection was diagnosed within 15 days, whereas rejection occurred in only 15% of patients who remained continuously below the cutoff levels (Cox-regression: relative risk 4.7, P=0.019). In 68% of the patients who exceeded serum sICAM-1 cutoff levels, acute rejection was diagnosed within 10 days (Figure 2; curve I), whereas rejection occurred in 26% of patients who continuously remained below the cutoff levels (Figure 2; curve II). When patients exceeded serum sICAM-1 cutoff levels, the relative risk for rejection was 4.8 (P=0.009). By the use of ANOVA, a significant correlation was found between serum sICAM-1 and biliary IL-8 levels during the first postoperative month (P=0.001); however, multivariate Cox-regression analysis showed that both serum sICAM-1 and biliary IL-8 were independently related to acute liver graft rejection (P=0.03 and P=0.05, respectively).

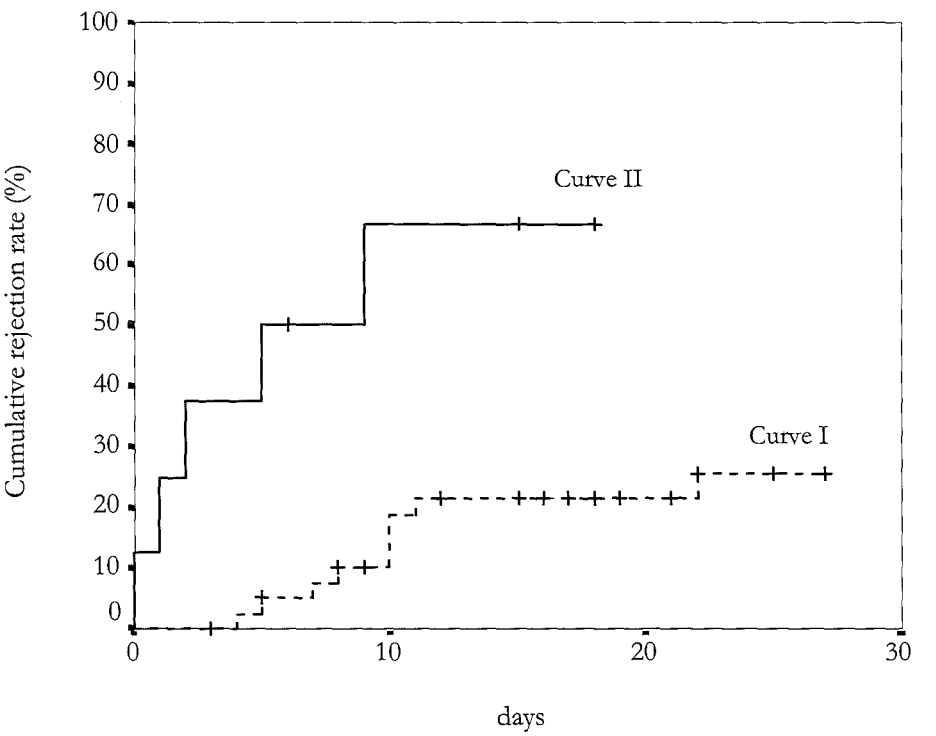

Figure 2. From day 0, each patient was followed until sICAM-1 exceeded the cutoff level that was computed for each postoperative day. The cumulative percentage of rejection as long as the cutoff level was not exceeded is shown in curve I. When the cutoff level was exceeded, the cumulative percentage of rejection after that timepoint is shown in curve II. Curves I and II therefore represent the rejecton probability before and after the cutoff level was exceeded.

Discussion

Differentiation between acute rejection and infection remains a challenging problem after liver transplantation. The results from this study demonstrate that daily monitoring of sICAM-1 in serum could improve differentiation between rejection and infection and may lead to earlier recognition of rejection. Furthermore, biliary IL-8 is also specifically increased before diagnosis of rejection. Nevertheless, daily measurements of cytokines in T-tube bile samples do not provide rejection markers with higher specificity compared with serum cytokines. Therefore, the diagnostic advantages of biliary drainage (e.g., radiographic imaging of the bile ducts and postoperative monitoring of bile production, including measurements of bilirubin and cytokine patterns) do not outweigh the risk of T-tube placement (e.g., biliary leakage).

Acute rejection is initiated by (in)direct recognition and activation of T cells and followed by the release of cytokines, which play a pivotal role in mediating the inflammatory allo-response. Administration of the IL-2R antagonist basiliximab inhibits activated T cells; therefore, the release of cytokines and serum and biliary cytokine levels may be influenced. In our study, serum sIL-2R and IL-10 levels were significantly decreased in patients who received basiliximab, whereas other serum and biliary cytokine levels (sICAM-1, sTNF-RII, IL-6, and IL-8) were not affected. There has been no previous report on the effect of basiliximab with respect to serum cytokine patterns after liver transplantation.

Monocytes and activated T cells are the main sources of soluble TNF-RII (27), which is supposed to reflect the activation state of the TNF-α/TNF-receptor system (28). TNF-α and TNF-RII have been shown to be early nonspecific markers of liver graft rejection and infection (6-11). Our results support these findings, because sTNF-RII levels are increased compared with uneventful patients before rejection and organ space/SSI (Figure 1).

In this study, serum levels of the proinflammatory cytokine IL-6 were increased before rejection in patients with rejection during an episode of infection, whereas serum IL-6 was not significantly increased in patients with infection or rejection only. These findings do not necessarily contradict previous reports in which IL-6 levels after liver transplantation were found to be increased during and not before episodes of (steroid-resistant) rejection (7, 12, 13), lethal infection (7), and cytomegalovirus disease (12). Furthermore, our results show that serum levels of IL-6 are remarkably decreased during treatment with high-dose methylprednisolone (Figure 2). Because serum levels of IL-6 were significantly decreased in patients who received triple therapy as initial immunosuppression, we could not exclude an effect of triple therapy as a confounding factor.

Effects of IL-8 on human neutrophil function are chemotaxis, enzyme release, and expression of adhesion molecules (29). Previous reports have shown that in liver allograft recipients, serum IL-8 is increased during acute rejection (15), bacterial

infection (6, 7, 15), and cytomegalovirus disease (15). In our study, however, serum levels of IL-8 were not significantly increased at the onset of infectious complications, whereas IL-8 levels in bile were specifically increased at the onset and before episodes of acute rejection (Figure 1). Biliary levels of IL-8 are approximately 10- to 1,000-fold higher compared with serum levels, indicating that IL-8-producing cells, such as Kupffer cells and vascular endothelium, actively release IL-8 into bile in contrast with other cytokines (sTNF-RII, IL-6, and sICAM-1) measured in bile after liver transplantation. There have been no previous reports on IL-8 measurements in bile after liver transplantation.

Activated T cells are characterized by increased expression of ICAM-1 on the cell surface and also release a soluble form (30). Previous studies showed that serum sICAM-1 is a marker of liver graft rejection (17-23), although sICAM-1 was also elevated during episodes of infection (20-23). In accordance with those studies, we show that serum sICAM-1 is significantly increased before and at the onset of liver graft rejection; however, we have not found an increase of serum sICAM-1 before infectious complications. There are several possible explanations for this discrepancy. In this study we excluded interference of increased cytokine levels from previous episodes of rejection and infection and controlled for changes in normal values by time-dependent Cox-regression analysis. Differences in type and grade of infection may be another explanation. In this study most patients experienced gram-positive infections (n=12); however (high-grade) gram-negative infections occurred in seven patients (including one patient with lethal infection).

With respect to biliary sICAM-1 in this study, no significant differences were found among patients with rejection, infection, and an uneventful course. One report (17), however, showed that biliary sICAM-1 during rejection was significantly elevated compared with other complications. A possible explanation for discrepancy with our results may be that we detected remarkable higher levels of biliary sICAM-1 not only in patients with rejection but also in patients with infection and an uneventful course (data not shown).

In summary, this study shows that biliary cytokines do not improve early differentiation between rejection and infection after liver transplantation. Moreover, our study emphasizes the clinical relevance of serum sICAM-1 as a specific marker of acute liver graft rejection. Daily monitoring of serum sICAM-1 can identify patients at risk for acute rejection, and therefore rejection may be recognized earlier in those patients.

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Part II

In vitro cytokine production profiles in liver transplant patients

Warlé MC, Farhan A, Metselaar HJ, Hop WCJ, Van der Plas AJ, Kap M, De Rave S, Kwekkeboom J, Zondervan PE, IJzermans JNM, Tilanus HW, Pravica V, Hutchinson IV, Bouma GJ.
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Are cytokine gene polymorphisms related to *in vitro* cytokine production profiles?
Liver Transplantation 2003, 9: 170-181.

Part II

Chapter 4

In vitro production of TNF-α and IL-13 correlates with acute liver transplant rejection

Individuals may differ in their capacity to produce cytokines. Since cytokines play a keyrole in allograft rejection, we investigated whether interindividual differences in cytokine production by *in vitro* stimulated PBMC are related to the occurrence of acute liver transplant rejection. Our study group comprised 49 liver transplant recipients and 30 healthy individuals. Rejection, which occurred within one month after liver transplantation, was defined in 22 patients ("rejectors") as biopsy-proven rejection, treated with high dose prednisolone. Patients who never experienced rejection episodes were termed as "non-rejectors" (n=27). PBMC of healthy individuals and of liver transplant recipients, collected late after transplantation (mean 3.5 years), were cultured in the presence of Concanavalin A. The production of TNF-α, IFN-γ, IL-10, and IL-13 was measured in supernatant after 1, 2, 3, 4, and 7 days of cell culture. In cell culture, stimulated PBMC of rejectors were found to produce significantly higher levels of TNF-α, while there was a trend towards higher production of IFN-γ and IL-10 as compared to non-rejectors. After grouping patients into high or low cytokine producers based upon reference levels of the healthy individuals using multivariate analysis it was found that occurrence of acute liver transplant rejection correlated to high production of TNF-α and low production of IL-13. After stimulated cell culture PBMC of liver transplant recipients show a differential production of TNF-α and IL-13 which is correlated with the occurrence of acute liver transplant rejection.

Introduction

Immunosuppressive regimens being used to prevent rejection after transplantation are associated with increased risks of opportunistic infections, malignancy, metabolic complications and heart- and vascular diseases (1-4). Despite immunosuppressive therapy, acute allograft rejection following liver transplantation occurs in 40% to 70% of patients (3-5). Considering the large variation in susceptibility for rejection between patients and the adverse effects of immunosuppressive agents, patients may benefit from an immunosuppressive scheme based upon their individual needs. Patients who are most likely to reject would require greater immunosuppression while patients less likely to reject could be treated with less immunosuppression. To this end assessment of the risk of acute liver allograft rejection would be required. In the present study we investigated in liver transplant recipients whether inter-individual differences in cytokine production are related to the occurrence of acute rejection episodes.

Cytokines are involved in rejection of human liver grafts (6-9). Th1-type cytokines including IL-2, TNF-α and IFN-γ, mediate cellular immune responses and are pro-inflammatory, while Th2-type cytokines like IL-4 and IL-10 have been shown to inhibit the development and function of Th1-cells, suppress inflammation and enhance humoral pathways of the immune response. Th1-cytokines are mainly involved in allograft rejection, by upregulation of MHC class I and II expression, stimulating macrophage function (TNF-α and IFN-γ), by endothelial cell activation, up-regulation of cell adhesion molecules and facilitating the recruitment and activation of leukocytes (TNF-α) (10, 11). Plasma levels of TNF-α are raised during acute rejection episodes following heart (12, 13), kidney (14, 15) and liver (16) transplantation. Conversely, evidence from animal transplant models has suggested a role for Th2-cytokines like IL-4 and IL-10 in promoting graft survival (17, 18). Their mechanism of action would be in down-regulating pro-inflammatory cytokine production. IL-10 may also down-regulate T-cell activation by decreasing MHC class II and B7 expression on antigen-presenting cells (19). However, others have provided evidence supporting a role of IL-10 in rejection (20) presumably through promoting B-cell mediated humoral responses (21-23).

Between individuals differences exist in the capacity to produce cytokines. It has been reported that levels of cytokine production are associated with polymorphisms in cytokine genes. For several cytokines like TNF-α, IFN-γ, TGF-β and IL-10 polymorphisms have been described which are associated with the level of *in vitro* cytokine production after stimulated cell culture of PBMC (24-28). In the present study we investigated whether PBMC of liver transplant recipients and healthy individuals differ in their functional capacity to produce cytokines in stimulated cell culture and whether inter-individual differences are related to the occurrence of

acute liver transplant rejection. To this end, PBMC of liver transplant recipients and, as a control, healthy individuals were cultured in the presence and absence of Con A. The level of *in vitro* cytokine production was determined for the cytokines TNF-α, IFN-γ, IL-10 and IL-13. TNF-α and IFN-γ were measured as Th1-type, pro-inflammatory cytokines, which have been shown to be involved in allograft rejection. Since the role of IL-10 in allograft rejection remains ambivalent, we also tested IL-13, of which the role in allograft rejection is not yet known, as a typical Th2-type cytokine (29, 30).

Materials and methods

Patients

Between 1992 and 1998, 130 orthotopic liver transplantations in 120 patients were performed at the Erasmus Medical Center Rotterdam. In this study, all patients were enrolled who paid a routine visit to the Transplant Outpatients' Clinic between September 1998 and December 1998 and who had been transplanted at least ten months previously. Thus, 49 patients were consecutively enrolled; 22 of them had experienced acute rejection after liver transplantation and 27 of them had not. The study was approved by the Medical Ethical Committee of the Eramus Medical Center Rotterdam and blood was collected after informed consent. Blood was collected after transplantation between 10 months and 6.7 years (mean 3.5 years) from date of transplant. Rejection, which occurred within one month after liver transplantation, was defined in 22 patients ("rejectors") as biopsy-proven rejection, Snover criteria ≥ 2 (31), and treated with three consecutive intravenous daily doses of 1000 mg prednisolone. Patients who never experienced rejection episodes were termed as "non-rejectors" (n=27). Initial immunosuppressive therapy included cyclosporine A (CsA), prednisone, and azathioprine in 46 patients, 3 patients received either Tacrolimus (FK506) and prednisone (n=1) or CsA and prednisone (n=2). Within three months after transplantation all patients were on maintenance immunosuppressive therapy; CsA (target level 100-200 ng/ml, *n*=48) or Tacrolimus (target level 5-10 ng/ml; n=1) and 7.5 mg prednisone. Typing of patients for HLA class I and II was performed using complement-dependent cytotoxicity and DNA-based typing methods (Tissue Typing Reference Laboratory, Department of Immunohematology and Blood Transfusion, LUMC, Leiden, The Netherlands). Thirty healthy volunteers (18 males/12 females) of the Rotterdam Blood Bank population served as a control group. These individuals were matched for age (mean age 43.7 years, range 22 to 64) with the patient population. The groups of nonrejectors and rejectors were comparable for sex, age, ethnicity, immuno-suppressive therapy, and indication for liver transplantation (see Table 1). The presence

of HLA-DR mismatches was correlated with the occurrence of liver transplant rejection (P=0.045), whereas mismatches at the HLA-A or HLA-B loci were not.

Table 1. Patient characteristics

	Nonrejectors (n=27)	Rejectors (n=22)
Gender (M/F)	12/15	12/10
Age (mean years and range)	46.5 (23-66)	46.8 (19-63)
Ethnicity		
Caucasoid/non-Caucasoid	21/6	21/1
Initial immunosuppression		
CsA/Prednisone/Azathioprine	25	21
CsA/Prednisone	1	1
Tacrolimus/Prednisone	1	0
Indication for liver transplantation		
Acute hepatic failure	7	4
Cryptogenic cirrhosis	4	1
HBV/HCV cirrhosis	3	6
PBC/PSC	9	7
Alcoholic cirrhosis	2	3
α-1 antitrypsin deficiency	1	0
Criggler najjar	1	0
Wilson	0	1
HLA-mismatches		
HLA-A (0/1)[a]/2	15/12	11/11
HLA-B (0/1)[b]/2	10/17	7/15
HLA-DR (0/1)[c]/2[d]	14/13	5/17

[a] 5 nonrejectors (0 rejectors) were completely matched for HLA-A

[b] 1 nonrejector (0 rejectors) was completely matched for HLA-B

[c] 1 nonrejector and 1 rejector were completely matched for HLA-DR

[d] Fisher Exact test P=0.045, for all other patient characteristics rejectors and nonrejectors did not significantly differ from each other.

In vitro cytokine production

PBMC were isolated from blood samples by centrifugation over a Ficoll gradient (Ficoll-Paque Research Grade, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cells collected from the interphase were washed twice, taken up in RPMI 1640 medium (Dutch modification, Gibco BRL, Life Technologies Ltd., Paisley, Scotland) supplemented with three mM L-glutamine (Gibco) and 10% pooled human serum and stored overnight at 37°C in a humidified atmosphere containing 5% CO_2. [Pooled serum used in this study consisted of human sera that were heat-inactivated (20 min,

56°C) and screened to exclude the presence of HLA alloantibodies. Moreover, sera were tested in a mixed lymphocyte culture assay using three different responder-stimulator combinations and excluded from the pool if inhibitory or stimulatory effects on proliferation were observed. Subsequently, PBMC were cultured in RPMI 1640 medium with L-glutamine and 10% pooled human serum in the absence and presence of 10 μg mL^{-1} Concanavalin A (Con A, Sigma Biosciences St Louis, MO, USA) at 5×10^5 mL^{-1} in 100 μl replicates in 96-well U-bottom plates (Costar tissue culture treated polystyrene, Corning Inc., Corning, NY, USA). Con A was chosen as the universal *in vitro* stimulus since Con A will provide a suboptimal stimulation for cytokine production (32). Initially, five separate cultures were set up in order to harvest culture supernatants at day 1, 2, 3, 4 and 7 after onset of cell culture. Culture supernatants were stored at -20°C until cytokines were measured. After thawing culture supernatants, TNF-α levels were determined by an automated chemi-luminescent enzyme immunoassay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA), whereas IFN-γ, IL-10 and IL-13 levels were measured using conventional ELISA assays (Pelikan Compact Human Elisa Kit, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) according to the manufacturer's instructions. Cytokine concentrations were calculated by reference to manufacturer supplied cytokine standards and expressed in pg mL^{-1}.

Statistical analysis

Differences in mean cytokine (TNF-α, IFN-γ, IL-10 and IL-13) production measured in supernatants at consecutive days after onset of cell culture between nonrejectors, rejectors and healthy individuals were analyzed by analysis of variance (ANOVA) for repeated measurements (BMDP Statistical Software Inc., Berkeley, CA, USA) (33). For this analysis data were transformed logarithmically in order to obtain approximate normal distributions. Further analysis were done by grouping patients into high or low cytokine producers. Patients were grouped into high or low producers when their maximum levels of cytokine production in response to Con A were respectively above or below the median level of maximum cytokine production by healthy individuals. Univariate analysis of differences in the number of high or low cytokine producers in comparing nonrejectors and rejectors was performed using the Fisher exact test for 2 × 2 tables. In a multiple logistic regression analysis the variables most predictive of acute liver transplant rejection were determined by logistic regression with backward elimination. P=0.05 (two-tailed) was considered the limit of significance.

Results

Differential *in vitro* cytokine production in liver transplant recipients

PBMC of healthy individuals and liver transplant recipients were cultured in the presence and absence of Con A, to examine *in vitro* cytokine production. Cell culture supernatant was harvested after 1, 2, 3, 4 and 7 days of culture and cytokine production was measured as described in the Materials and Methods section. In unstimulated cell culture TNF-α, IFN-γ, IL-10 and IL-13 was produced at low to undetectable levels (data not shown). Since PBMC of liver transplant recipients were tested for their *in vitro* cytokine production at a time-point after liver transplantation (mean 3.5 years), we determined whether immunosuppressed liver transplant recipients (n=49) and healthy individuals (n=30) differed in their capacity to produce cytokines *in vitro*. Using ANOVA, it was found that the group of liver transplant recipients as a whole and the group of healthy individuals did not differ significantly regarding mean TNF-α, IFN-γ, IL-10 and IL-13 levels in response to Con A stimulation during the period of cell culture. Subsequently, we assessed whether the occurrence of acute liver transplant rejection was related to the level of *in vitro* cytokine production, comparing *in vitro* cytokine production by PBMC of non-rejectors (n=27) and rejectors (n=22) (Figure 1). ANOVA showed that the mean level of TNF-α production during the culture period was significantly higher in rejectors as compared to non-rejectors (P=0.045). For IFN-γ and IL-10, rejectors tended to produce higher levels than non-rejectors (respectively, P=0.090 and P=0.069). Concerning the *in vitro* production of IL-13 no significant differences were found between non-rejectors and rejectors (P=0.868).

Classification of liver transplant recipients into high or low cytokine producers

For each patient or healthy individual the maximum level of cytokine production measured during the period of cell culture, was determined at the day of highest cytokine production. Kinetics of cytokine production differed between the cytokines studied; maximal TNF-α and IFN-γ production was usually reached after three or four days of cell culture, maximal IL-10 production after two, three, or four and IL-13 production after four or seven days of cell culture. Patients were grouped into *high* or *low* producers according to their maximum level of cytokine production, using median levels of maximum cytokine production by healthy volunteers as reference. *High* producers were defined as those patients producing TNF-α, IFN-γ, IL-10 or IL-13 above the median level (respectively 418, 3570, 45, or 122 pg/ml) of cytokine production by healthy individuals, whereas *low* producers produced cytokines below the median. When the relation between the number of high or low producer-patients and the occurrence of acute liver transplant rejection was analyzed

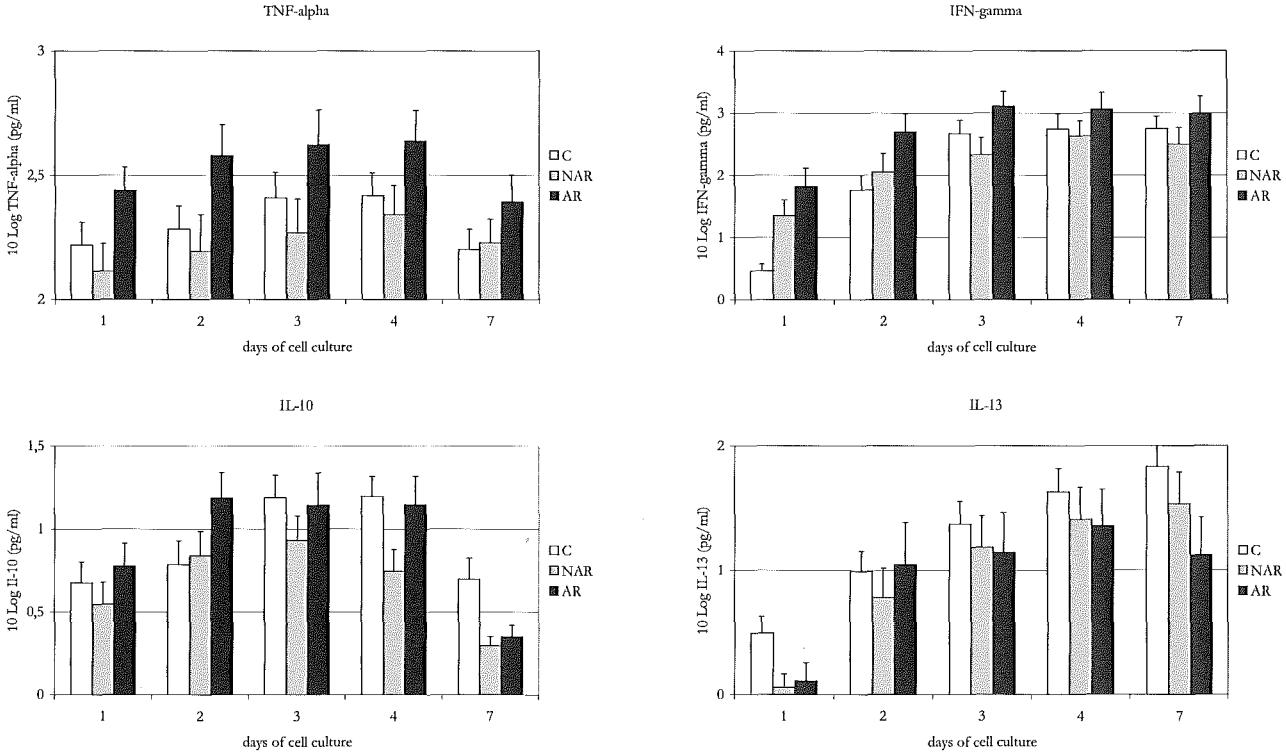

Figure 1. Differential *in vitro* cytokine production in liver transplant recipients. Cytokine production by Con A stimulated PBMC of 30 healthy individuals (C), 27 nonrejectors (NAR) and 22 rejectors (AR) measured at several days of stimulated cell culture. The production of TNF-α, IFN-γ, IL-10, and IL-13 was measured in cell culture supernatant harvested at 1, 2, 3, 4 and 7 days after onset of culture. Data given in figures are means ± standard error. Rejectors produced, during the period of stimulated cell culture, higher levels of TNF-α (P=0.045) and they showed a trend towards higher production of IFN-γ (P=0.090) and IL-10 (P=0.069) as compared to nonrejectors. PBMC of rejectors and nonrejectors did not differ in the production of IL-13 (P=0.868).

univariately, we found a significant correlation for TNF-α, relating acute liver transplant rejection to high TNF-α producers (P=0.039). No significant correlation was found between *high/low* production of IFN-γ, IL-10 or IL-13 and the occurrence of acute liver transplant rejection in these univariate analyses (Table 2).

Table 2. Capacity of *in vitro* cytokine production and acute liver transplant rejection

		Liver transplant recipients[a]		
	In vitro capacity	Nonrejectors	Rejectors	*P* value[b]
TNF-α	*High*	10	16	0.039*
	Low	15	6	
IFN-γ	*High*	13	12	0.359
	Low	14	6	
IL-10	*High*	7	11	0.136
	Low	20	11	
IL-13	*High*	12	7	0.752
	Low	12	10	

[a] In total 49 liver transplant recipients were tested for *in vitro* cytokine production. Some cases are missing as TNF-α, IFN-γ, IL-10, and IL-13 production was measured in respectively 47, 45, 49 and 41 liver transplant recipients, due to insufficient cell culture supernatant.
[b] Fisher Exact test computed for 2 × 2 tables, *significant at < 0.050 level.

TNF-α and IL-13 production is correlated to acute liver transplant rejection

In order to determine the most important variables correlating with the occurrence of acute liver transplant rejection, we performed a multiple logistic regression analysis, with *high/low* production of TNF-α, IFN-γ, IL-10, IL-13 and HLA-DR mismatches (see Table 1) as independent variables. *High/low* production of IFN-γ and IL-10 did not significantly contribute to predict the occurrence of acute rejection, neither did the number of HLA-DR mismatches. TNF-α (P=0.012) and IL-13 (P=0.056) production appeared to correlate independently with acute liver transplant rejection, although the latter was of borderline significance. Patients classified as *high TNF-α/ low IL-13* producers suffered in 86% of cases acute rejection as compared to none of the *low* TNF-α/*high* IL-13 producers and 22% of all *low* TNF-α producers, whereas *high* TNF-α/*high* IL-13 producers experienced in 44% of cases acute rejection after liver transplantation (Table 3, P=0.027).

Table 3. TNF-α production in combination with IL-13 production correlates with acute liver transplant rejection

	TNF-α and IL-13 production[a]			
TNF-α	High	High[b]	Low[c]	Low[d]
IL-13	Low	High	Low	High
Nonrejectors	1	9	11	3
Rejectors	6	7	4	0

[a] Fisher Exact test computed for 4×2 table: p=0.027. Pairwise comparison with *high* TNF-α/*low* IL-13 producers (Fisher Exact test for 2×2 tables).
[b] P=0.087.
[c] P=0.020.
[d] P=0.033.

Discussion

In the present study we investigated whether patients who experienced a rejection episode shortly after liver transplantation differ from patients without rejection in the functional capacity of their PBMC to produce cytokines in stimulated cell culture. Our results show that patients classified as *high TNF-α/low IL-13* producers most often rejected their liver allograft, whereas *low TNF-α/high IL-13* never rejected. Apparently, between PBMC of rejectors and nonrejectors a differential capacity to produce TNF-α is still measurable (mean 3.5 years after transplantation), long after the rejection event has taken place. In our patients rejection episodes typically occur within the first month after liver transplantation. Once they have passed this first critical month, repeated rejection is rare. Also, maintenance immunosuppressive therapy is installed within three months after transplantation, again long before the time of blood-sampling, which favors the argument that differential cytokine production between rejectors and nonrejectors is a predisposing factor rather than a consequence of rejection. Evidence for this argument also comes from preliminary data obtained after testing a small number of patients (8 rejectors and 8 nonrejectors) from which liquid nitrogen stored PBMC were available pre- and post-transplantation (results not shown). After Con A stimulation, mean levels of TNF-α production tended to be higher in rejectors than nonrejectors at each day of cell culture, both in the pre-transplantation and the post-transplantation obtained samples. Similar data were recently obtained by Bathgate *et al.* (34), who described higher TNF-α production by rejectors than nonrejectors after *in vitro* stimulation of pre-transplantation obtained blood samples by LPS. Another study found that PBMC obtained at the day of transplantation from patients who went on to develop kidney graft rejection produced more TNF-α and IL-2 after *in vitro* donor mixed lymphocyte culture than patients who experienced no rejection (35). In this study it is not excluded

that differences in the number of HLA mismatches between patients with and without rejection may have influenced the observed cytokine production.
Our results are in accordance with the established role of TNF-α in allo-reactions such as upregulation of MHC- and adhesion molecules and activation of leukocytes and endothelial cells (10, 11). Although no association of IL-13 with acute rejection was found in the univariate analysis, the multivariate regression analysis revealed that in combination with TNF-α the level of IL-13 production correlated significantly with acute liver transplant rejection. In patients classified as *high* TNF-α producers, graft rejection occurred more often if they were *also low* producers of IL-13, whereas *high* TNF-α/*high* IL-13 producers were less likely to reject. These findings may imply that acute liver graft rejection is less likely to occur when the production of TNF-α during the allo-response is inhibited by high levels of IL-13. In previous reports, the inhibitory effect of IL-13 on inflammatory cytokine production *in vitro* by stimulated human PBMC (36) and monocytes (29) has been described. Thus far no studies investigated the role of IL-13 in allograft rejection. *In vitro* production of another Th2-cytokine, IL-10, did not significantly correlate with acute liver transplant rejection, although our results showed a tendency of lower *in vitro* IL-10 production by PBMC of nonrejectors. Also in other studies IL-10 seems to be positively correlated to human graft rejection (22, 23, 37), while a role for IL-10 in transplant tolerance mainly holds for animal models (17, 18). In our study PBMC of rejectors produced higher levels of IFN-γ than non-rejectors, although no statistical significance was reached. Interestingly, a similar study found the occurrence of acute kidney graft rejection correlated to high levels of IFN-γ in stimulated cell culture (38). Moreover, the same group found that pre-transplantation high IFN-γ as well as high IL-10 levels produced after *in vitro* donor mixed lymphocyte cultures, were significantly correlated to acute kidney graft rejection (39).
The role of histocompatibility between donor and recipient in human liver transplant rejection remains uncertain after clinical studies, which either confirm or deny the influence of HLA class I or HLA class II matching on liver graft survival (40-45). The discrepancies between these studies may result from differences in the level of immunosuppression as high dosage may obscure the effect of HLA-matching on the occurrence of acute liver transplant rejection (46, 47). In the present study, the influence of HLA matching was tested univariately and HLA-DR mismatching appeared to be significantly associated with acute liver transplant rejection, whereas HLA-A and –B matching did not. Subsequently, we assessed the role of HLA-DR mismatches in combination with *high*/*low* TNF-α, IFN-γ, IL-10 and IL-13 production in acute liver transplant rejection by multivariate logistic regression analysis. Then, neither IL-10 and IFN-γ production, nor the presence of mismatches at the HLA-DR locus were found to correlate independently with acute liver transplant rejection, whereas TNF-α production did and it was strongly suggested that IL-13 plays a

role. These findings suggest that HLA incompatibility between donor and liver transplant recipient may trigger the allo-response. Then, actual cytokine levels, in particular TNF-α and IL-13, which are associated with respectively enhancement of or protection from rejection in the present study, determine whether the allo-response results in clinical signs of acute rejection.

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Chapter 5

Are cytokine gene polymorphisms related to *in vitro* cytokine production profiles?

Currently, there is much interest in the genetic basis for diseases or disease manifestations and, in particular, in whether they are related to cytokine gene polymorphisms. It has become accepted to denote such single-nucleotide polymorphisms of cytokine genes by their presumed association with high or low *in vitro* cytokine production. In this article, we analyze the relationship between cytokine gene polymorphisms and *in vitro* tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin (IL)-10 and IL-13 production, both in liver transplant recipients and in healthy volunteers. The evaluated cytokine gene polymorphisms involved TNF-A –308; TNF-d3; IFN-G +874; IL-10 –1082, -819, -592; and IL-13 +2043, and –1055. For healthy volunteers, we observed a relationship between polymorphisms of TNF-d3 and IL-10 –1082 with *in vitro* production of TNF-α and IL-10, respectively, whereas no significant associations were found for the other tested cytokine gene polymorphisms. For liver transplant recipients, no significant relationship could be established between any of the cytokine gene polymorphisms and *in vitro* production of corresponding cytokines. Also, we reviewed the literature for the association between cytokine gene polymorphisms and *in vitro* cytokine production in various patient groups and healthy volunteers. We found that the cellular sources, from which the cytokines were released into the culture supernatant, were different between studies. They were either whole blood, isolated monocytes, or PBMC. Also, the *in vitro* incubation protocol varied to a great extent between studies. This applied for the used *in vitro* stimulant, and the length of the incubation period. Moreover, the study populations were either healthy individuals or very diverse patient groups. Therefore, it was impossible to evaluate whether *in vitro* cytokine production profiles really can be deduced from a particular cytokine gene polymorphism. Given the inconclusive findings, we propose to set up a multicenter workshop in which the relationship between certain cytokine gene polymorphisms and *in vitro* cytokine production is analyzed, using an identical *in vitro* cell culture system and study population. Furthermore, we suggest that cytokine gene polymorphisms be described by their localization within the gene or gene-promoter, rather than by their presumed *in vitro* cytokine production profile, to properly evaluate the relationship between cytokine gene polymorphisms and disease manifestations.

Introduction

Cytokines are involved in many diseases and, consequently, many studies have been conducted to investigate whether a genetic basis for these diseases may be found in polymorphisms of cytokine genes. For example, the tumor necrosis factor (TNF)-A –308 polymorphism was found to be associated with severe rejection after heart, kidney, and liver transplantation (1-4) and with a wide variety of diseases, such as primary sclerosing cholangitis (5, 6), autoimmune hepatitis (6, 7), cerebral malaria (8), mucocutaneous leishmaniasis (9), and asthma (10). These associations were not always confirmed; for example, some studies could not show a relationship between the TNF-A –308 gene polymorphism and liver graft rejection (11-13).

The reasoning behind the proposed involvement of cytokine gene polymorphisms in diseases or disease manifestations is that they may influence *in vivo* cytokine levels. This presumption is based on the observation that individuals differ with respect to the level of cytokine production after *in vitro* culture of their cells and that these differences may be attributed partly to single-nucleotide or microsatellite polymorphisms of their cytokine genes. It is becoming popular linguistic usage to name individuals with a particular cytokine genotype "high" or "low" producers, after the presumed relationship between that genotype and the corresponding *in vitro* cytokine production profile (3, 13, 14).

In this study, we investigated whether *in vitro* cytokine production profiles really can be deduced from given single-nucleotide or microsatellite polymorphisms of cytokine genes. Also, we analyzed whether similar relationships between *in vitro* cytokine production and cytokine gene polymorphisms may be found in healthy individuals and in patients. One may imagine that healthy individuals and patients differ in their *in vitro* cytokine response to identical stimuli, for example, because of the fact that the immune system of most patients may be in an activated state because of their disease or, alternatively, be under the influence of immunosuppression in the case of transplant recipients. We restricted ourselves to those polymorphisms for which we obtained *in vitro* production and cytokine genotype data; we analyzed *in vitro* production of TNF-α, interferon gamma (IFN-γ), and interleukin (IL)-10 and IL-13, with polymorphisms in corresponding cytokine genes, respectively TNF-A –308 and TNF-d3; IFN-G +874; IL-10 –1082, -819, and –592; and IL-13 +2043 and –1055.

Materials and methods

Study population

In this study, primary liver transplant recipients were consecutively enrolled who had undergone transplantation from 1992 onward and who were seen at the

transplantation outpatient clinic between September 1998 and December 1998. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam, and blood was collected after informed consent. The patients underwent transplantation at least 10 months before the date of blood collection. The total group was 44 patients (20 men, 24 women). Nineteen patients experienced biopsy-proven and treatment-requiring acute rejection after liver transplantation, and 25 of them did not. Initial immunosuppressive therapy included cyclosporine A (CsA) or tacrolimus (FK506) and prednisone, with or without azathioprine. Within 3 months after transplantation, all patients were on maintenance immunosuppressive therapy; CsA (target level, 100-200 ng/mL) or FK506 (target level, 5-10 ng/mL) and prednisone. Main indications for liver transplantation were PBC, PSC, acute hepatic failure, chronic hepatitis B and C, and alcoholic and cryptogenic cirrhosis. Thirty healthy volunteers (18 men, 12 women) of the Rotterdam Blood Bank population served as a control group. These individuals were matched for age (mean age, 43.7 years; range, 22-64) with the patient (mean age, 47.4 years; range, 20-66) population.

In vitro cytokine production

The *in vitro* stimulation protocol was performed as described previously (15). Briefly, PBMCs were isolated from blood samples of liver transplant recipients and healthy volunteers, taken up in RPMI 1640 medium (Dutch modification, Gibco BRL, Life Technologies Ltd, Paisley, Scotland) supplemented with 3mmol/L L-glutamine (Gibco) and 10% pooled human serum and stored overnight at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, the PBMCs were cultured in RPMI 1640 medium with L-glutamine and 10% pooled human serum in the presence of 10 mg/mL Concanavalin A (Con A, Sigma Biosciences, St. Louis, MO) at 5×10^5/mL in 100 mL replicates in 96 well U-bottom plates (Costar tissue culture treated polystyrene, Corning Inc, Corning, NY). Replicate cultures were set up, and culture supernatants were stored at -20°C until cytokines were measured. TNF-α levels were determined by an automated chemiluminescent enzyme immunoassay (Immulite, Diagnostic Products Corporation, Los Angelos, CA), whereas IFN-γ, IL-10, and IL-13 levels were measured with conventional enzyme-linked immunosorbent assays (Pelikine Compact Human Elisa Kit, Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) according to the manufacturer's instructions.

Cytokine genotyping

DNA was isolated from blood samples of liver transplant recipients and healthy volunteers by the classic salting-out method, involving proteinase K digestion and ethanol precipitation. TNF-d3 microsatellite typing was performed by SDS-PAGE

as previously described (16). Amplification Refractory Mutation System (ARMS)-based polymerase chain reaction (PCR) methodologies were developed for the single-nucleotide substitutions at the positions –592, -819, and –1082 in the IL-10 gene, -1055 and +2043 in the IL-13 gene, -308 in the TNF-α gene, and +874 in the IFN-γ gene as previously described (17). Briefly, DNA was amplified in a 10 μL reaction, and final concentrations of reagents were 1× customized PCR master mix (Abgene, Epsom, UK), 5 μM specific primer mix (specific primer mix consists of 10 μM generic primer and 10 μM of one of the two allele-specific primers), 0.5 μM internal control primer mix (internal control primers are used to check for successful PCR amplification; these primers amplify a human growth hormone sequence), and 25-100 ng DNA. The protocol for the PTC-100 PCR machine (MJ Research, MA) was as follows: 95°C for 15 seconds, 65°C for 15 seconds, 65° for 50 seconds and 72°C for 40 seconds (10 cycles) and 95°C for 20 seconds, 59°C for 50 seconds and 72°C for 50 seconds (20 cycles). The amplified products were monitored by electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 mg/mL).

Statistical analysis

The relationship was analyzed between cytokine (TNF-α, IFN-γ, IL-10 and IL-13) production levels measured in supernatants 1 and 2 days after onset of cell culture and polymorphisms of the corresponding cytokine genes. When a particular cytokine genotype was present in few individuals, the presence or absence of a chosen allele was analyzed instead of the genotype. For example, the genotypes determined at position –308 in the TNF-A gene were distributed within the patient group as follows: homozygous AA present in one patient, heterozygous GA present in 19 patients, and homozygous GG present in 24 patients. Because the genotype AA was present in very few individuals, we grouped the AA and GA genotypes and compared them with the GG genotype. Regarding the other studied gene polymorphisms, the following genotype groups were compared: the presence or absence of the TNF-d3 allele, IFN-G +874 TT genotype versus the grouped IFN-G +874 AT and AA genotypes, IL-10 –1082 GG genotype versus IL-10 –1082 AG/AA genotypes, IL-13 +2043 GG genotype versus IL-13 +2043 AG/AA genotypes, and IL-13 –1055 CC genotype versus the grouped IL-13 –1055 CT/TT genotypes. Within the patient and healthy volunteer population, cytokine production levels for the different genotype groups were compared using the Mann-Whitney U test. This was done at both day 1 and day 2 after Con A stimulated cell culture. P=.05 (two-tailed) was considered the limit of significance.

Results

In this study, *in vitro* cytokine production by liver transplant recipients and healthy volunteers was measured at day 1 and day 2 after beginning of Con A-stimulated cell culture. For each cytokine, the *in vitro* production was related to gene polymorphisms of the corresponding cytokine. These results are displayed in Figures 1 through 4.

In vitro TNF-α production and TNF-A –308 gene polymorphism

No significant relationship was observed between TNF-α production and TNF-A –308 gene polymorphism, either for liver transplant recipients or healthy volunteers (Figure 1A). This applied to outcomes at both day 1 and 2 after Con A-stimulated cell culture.

In vitro TNF-α production and TNF-d3 gene polymorphism

Liver transplant recipients, who possessed a TNF-d3 allele, produced on average slightly more TNF-α than patients who were negative for TNF-d3, although no statistical significant difference in TNF-α production was reached (Figure 1B). Healthy individuals who were positive for TNF-d3 produced significantly more TNF-α than TNF-d3 negative healthy volunteers, which was evident after 1 day of Con A-stimulated cell culture (P=.026).

In vitro IFN-γ production and IFN-G +874 gene polymorphism

To evaluate whether polymorphisms in the IFN-γ encoding gene were related to *in vitro* IFN-γ production, we analyzed the IFN-G +874 polymorphism. Healthy volunteers of the TT-genotype produced on average more IFN-γ than AT/AA-genotyped individuals (Figure 2). This difference showed a trend toward significance after 1 day of cell culture (P=.064). For liver transplant recipients, no significant associations between *in vitro* IFN-γ production and the IFN-G +874 polymorphism were found.

In vitro IL-10 production and IL-10 –1082 gene polymorphism

Healthy volunteers who were typed as IL-10 –1082 GG produced on average significantly less IL-10 than did IL-10 –1082 AG/AA individuals (day 1, P=.040; day 2, P=.002; see Figure 3). For liver transplant recipients, no significant associations between *in vitro* IL-10 production and IL-10 –1082 polymorphism were found. The distribution of the IL-10 –1082 GG versus AG versus AA genotypes was identical to the IL-10 haplotype –1082, -819, -592; respectively GCC/GCC versus GCC/ACC, GCC/ACC, GCC/ATA versus ATA/ATA, ACC/ATA, ACC/ACC distribution, in liver transplant recipients and healthy individuals.

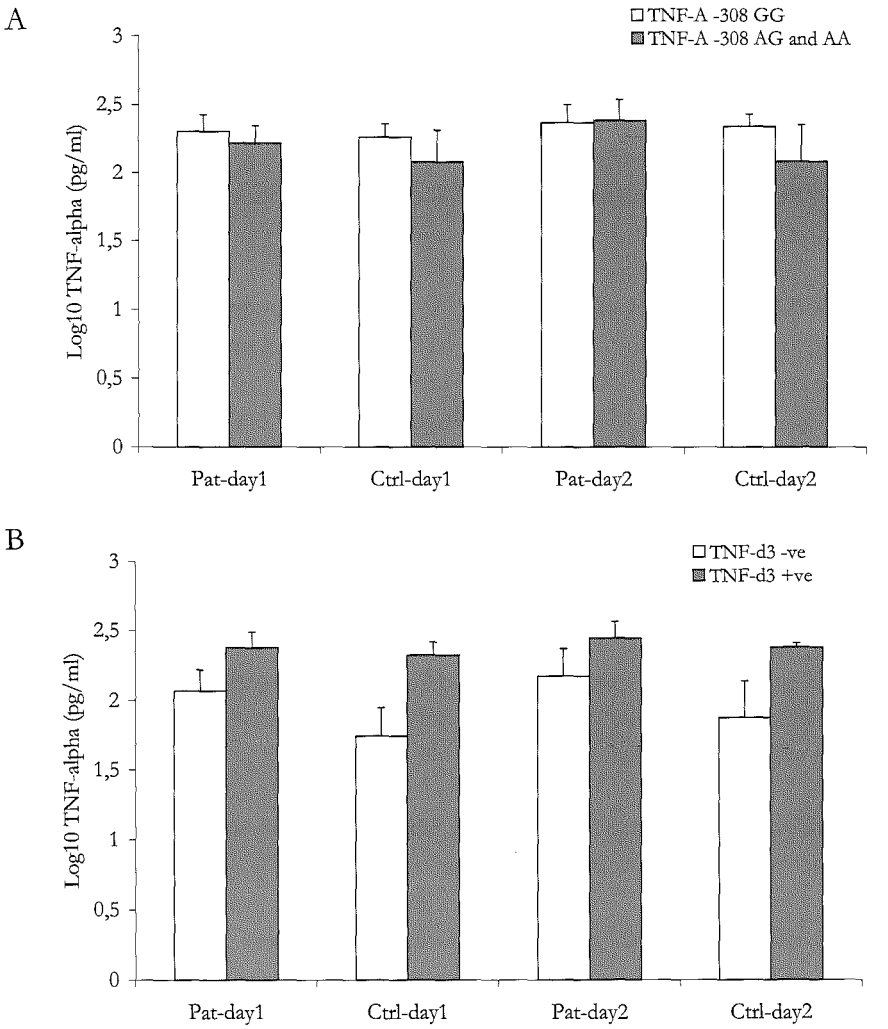


Figure 1. TNF-α production was measured at day 1 and 2 after onset of Con A-stimulated cell culture of fresh peripheral blood mononuclear cells. TNF-α production of liver transplant recipients (Pat) and healthy individuals (Ctrl) was related with (A) TNF-A –308 and (B) TNFd3 cytokine gene polymorphism. Displayed are mean TNF-α production data ± SEM after logarithmic transformation to obtain approximate normal distributions. (A) The distribution of the cytokine genotypes was as follows: TNF-A –308 GG: 22 patients and 23 healthy individuals; TNF-A –308 AG/GG: 19 patients and 7 healthy individuals. No significant associations between TNF-A –308 genotypes and *in vitro* TNF-α production were determined. This applied to patients and healthy individuals. (B) TNFd3 cytokine genotypes were distributed as follows: TNF-d3-ve: 14 patients and 6 healthy individuals; TNF-d3+ve: 26 patients and 23 healthy individuals. On average, TNF-d3+ve individuals produced more TNF-α than TNF-d3-ve individuals. However, in patients, the differential *in vitro* cytokine production never reached statistical significance, whereas healthy individuals showed a significantly higher (at day 1, P=.026) and a trend toward higher (at day 2, P=.071) TNF-α production than TNF-d3 –ve healthy individuals.

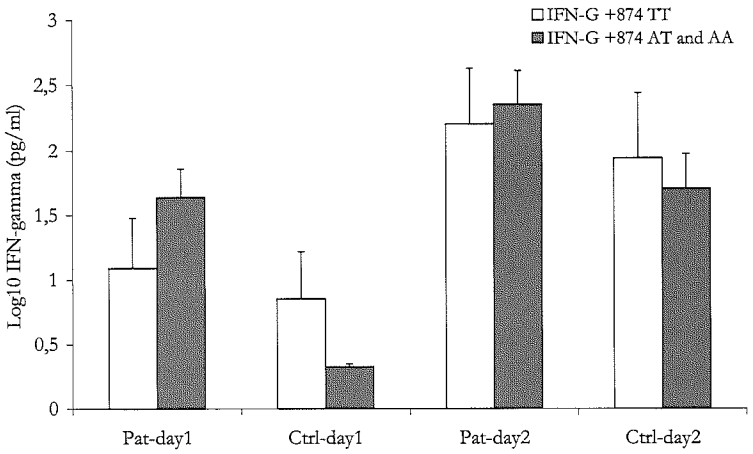


Figure 2. IFN-γ production was measured at day 1 and 2 after onset of Con A-stimulated cell culture of fresh peripheral blood mononuclear cells. IFN-γ production of liver transplant recipients (Pat) and healthy individuals (Ctrl) was related with IFN-G +874 cytokine gene polymorphism. The distribution of the cytokine genoptype was as follows: IFN-G +874 TT: 10 patients and 8 healthy individuals; IFN-G +874 AT/AA: 30 patients and 22 healthy individuals. In patients, IFN-G +874 AT/AA individuals on average produced more IFN-γ than IFN-G +874 TT individuals, although statistical significance was never reached. In healthy individuals, at day 1 of cell culture, IFN-G +874 TT individuals showed a trend (*P*=.064) toward higher *in vitro* IFN-γ production than IFN-G +874 AT/AA healthy individuals.

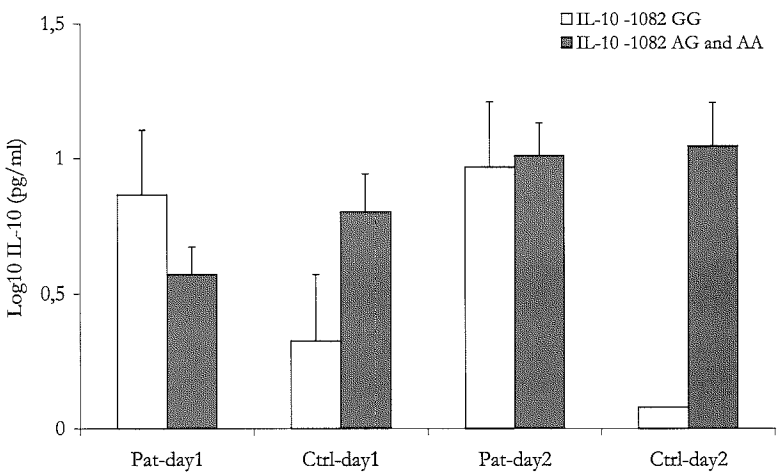


Figure 3. IL-10 production was measured at day 1 and 2 after onset of Con A-stimulated cell culture of fresh peripheral blood mononuclear cells. IL-10 production of liver transplant recipients (Pat) and healthy individuals (Ctrl) was related with IL-10 –1082 cytokine gene polymorphism. The distribution of the cytokine genotype was as follows: IL-10 –1082 GG: 12 patients and 8 healthy individuals; IL-10 –1082 AG/AA: 31 patients and 22 healthy individuals. In patients, no significant association between IL-10 –1082 genotype and *in vitro* IL-10 production was detected, whereas IL-10 AG/AA healthy individuals produced significantly more IL-10 at day 1 and 2 of cell culture (*P* values respectively, .040 and.002) than did IL-10 –1082 GG individuals.

In vitro IL-13 production and IL-13 +2043 gene polymorphism

IL-13 +2043 GG genotyped liver transplant recipients showed a trend toward higher IL-13 production after 2 days of Con A-stimulated cell culture compared with AG/AA patients (P=.058, see Figure 4A). Healthy volunteers with the GG-genotype produced on average slightly more IL-13 than AG/AA individuals, although statistical significance was not reached.

In vitro IL-13 production and IL-13 –1055 gene polymorphism

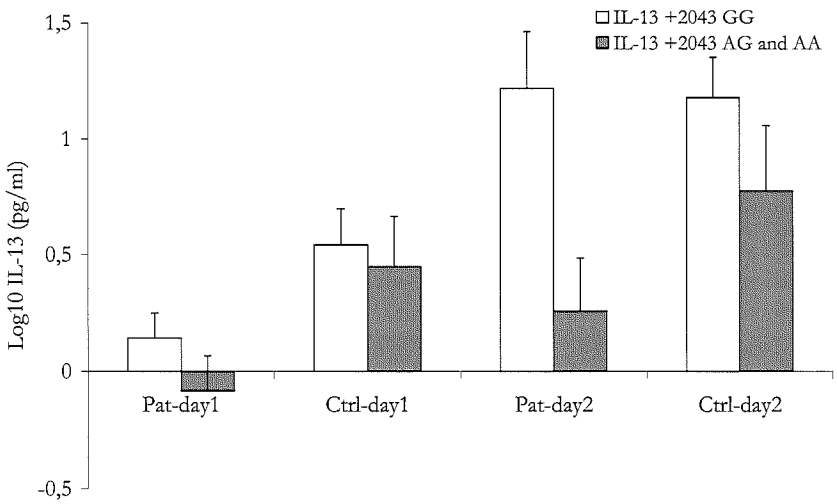
No significant differences in IL-13 *in vitro* production were detected between IL-13 –1055 CC- or CT/TT-genotyped individuals (Figure 4B). Because statistical significance was never reached, the results were only suggestive for differential *in vitro* IL-13 production comparing patients and healthy volunteers, related to the IL-13 –1055 polymorphism.

Discussion

In this study, we analyzed the association between single nucleotide or microsatellite polymorphisms of cytokine genes and *in vitro* production of the corresponding cytokines. We analyzed this relationship for liver transplant recipients and for healthy volunteers. Here, we discuss our data and review the literature for each cytokine gene polymorphism. In the reviewed literature, different groups of patients have been analyzed, as well as healthy volunteers.

An overview of the current literature describing the relationship between *in vitro* TNF-α production and TNF-A –308 gene polymorphism is given in Table 1. In these studies, different stimulation protocols have been applied using different cell culture systems, which involved either isolated monocytes, whole blood, or PBMC. In general, the TNF-A –308 AA or GA genotype is being termed the high TNF-α producing genotype (3, 13, 14). Originally, the presumed relation between the level of cytokine production and the TNF-A –308 gene-promoter polymorphism was based on the observation that the TNF-A –308 A +ve allele induced a higher level of gene transcription than the homozygous TNF-A –308 GG genotype (18), although this observation was not always confirmed (19). The hypothesized relationship between high TNF-α production and the GA/AA genotype was found (see Table 1) in a study involving LPS-stimulated whole blood samples obtained from healthy volunteers (20), one study in which the PBMC of a mixed population of IBD patients and healthy volunteers were stimulated with anti-CD3 and anti-CD28 (21), and in a recent small study of PHA-stimulated PBMC from paediatric renal transplant recipients (22). Other studies that either had similar stimulation protocols for whole blood samples (16, 23) or varying stimulation protocols for PBMC (24-27) could not confirm these results. Also our own data showed no association between TNF-α production and TNF-A –308 gene polymorphism.

A



B

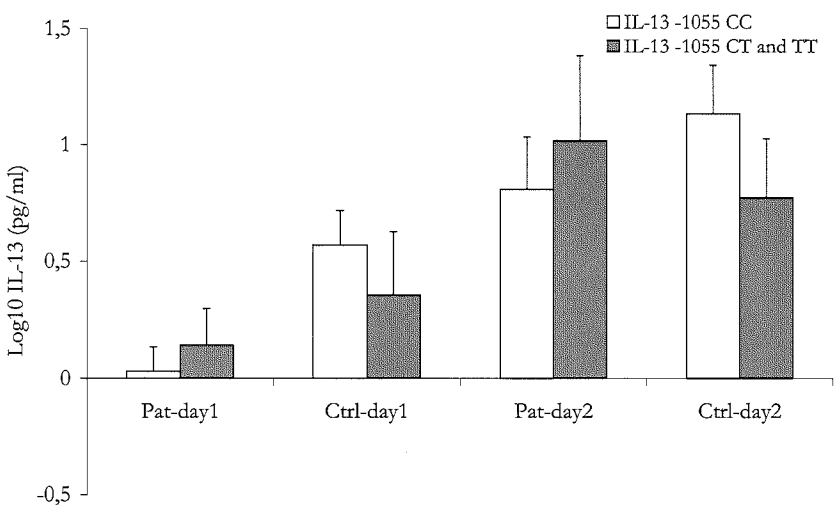


Figure 4. IL-13 production was measured at day 1 and 2 after onset of Con A-stimulated cell culture of fresh peripheral blood mononuclear cells. IL-13 production of liver transplant recipients (Pat) and healthy individuals (Ctrl) was related with (A) IL-13 +2043 and (B) IL-13 –1055 cytokine gene polymorphisms. (A) The distribution of the cytokine genotypes was as follows: IL-13 +2043 GG: 24 patients and 15 healthy individuals; IL-13 +2043 AG/AA: 10 patients and 13 healthy individuals. On average, statistical significance was never reached, whereas IL-13 +2043 GG patients, at day 2 of cell culture, showed a trend (P=.058) toward higher production than IL-13 +2043 AG/AA patients. (B) The distribution of genotypes was as follows: IL-13 –1055 CC: 19 patients and 17 healthy individuals; IL-13 –1055 CT/TT: 14 patients and 11 healthy individuals. It seemed that IL-13 –1055 CC healthy individuals produced on average more and IL-13 –1055 CC patients produced on average less IL-13 *in vitro* than IL-13 –1055 CT/TT individuals, however, statistical significance was never reached.

Table 1. Association between *in vitro* TNF-α production and TNF-A –308 cytokine gene polymorphism

Study population	Stimulation protocol[a]	Genotype	Results	Ref.
Healthy volunteers	Monocytes, LPS (250 pg/mL), 20 hr	GG genotype GA/AA genotype $P = 0.08$	2.88 ± 1.15 ng/mL[b] 3.33 ± 0.99 ng/mL n (total) = 74	29
Healthy volunteers	Monocytes, GM-CSF (10 ng/mL), and IFN-γ (5 ng/mL), 20 hr	GG genotype GA genotype $P = 0.79$	4.2 ng/mL (median), n = 29 5.9 ng/mL (median), n = 6	30
Heart transplant recipeints	Whole blood, endotoxin (5 u/mL), 4 hr	GG genotype GA/AA genotype P = not significant	427 (241-634) pg/mL[c], n = 14 274 (191-639) pg/mL, n = 8	16
Healthy volunteers	Whole blood, endotoxin (10 and 1000 ng/mL), 6 hr	GG genotype GA genotype AA genotype P = not significant	2.9 ± 1.8, 5.2 ± 2.4 pg/mL[b, d], n = 106 3.4 ± 2.6, 5.6 ± 2.6 pg/mL, n = 67 2.7 ± 1.7, 4.2 ± 1.4 pg/mL, n = 6	23
Healthy volunteers	Whole blood, LPS (1 ng/mL), 24 hr or (100 ng/mL), 3 hr	GG genotype GA genotype AA genotype $P < 0.05$, 0.16	521 (178-1307), 174 (57-323) pg/mL[e, f], n = 41 929 (480-1473), 203 (109-341) pg/mL, n = 16 n = 0	20
Healthy volunteers and IBD (UC and CD) patients	PBMC, anti-CD3 (1 μg/mL), and anti-CD28 (1 μg/mL), 48 hr	GG genotype GA genotype AA genotype $P = 0.014$ GG versus GA/AA genotype, $P = 0.032$	19,629 ± 7421 pg/mL[b], n = 26 24,923 ± 10,081 pg/ml, n= 13 33,525 ± 4033 pg/mL, n = 3	21
Healthy volunteers	PBMC, LPS (10 μg/mL), 4 hr	GG genotype GA genotype P = not significant	1050 ± 250 pg/mL[b, g] 1000 ± 25 pg/mL Exact n not given	27
MS patients	PBMC, LPS (10 μg/mL), 4 hr	GG genotype GA genotype P = not significant	750 ± 300 pg/mL[b, g] 600 ± 250 pg/mL Exact n not given	27
HIV-1 seronegative donors	PBMC, LPS, or PHA/IL-2 or PMA/ionomycin or enterotoxin B	GG genotype GA genotype AA genotype P = not significant	Data not given, n = 14 Data not given, n = 10 Data not given, n = 2	24
Sarcoidosis patients	PBMC, LPS (1 μg/mL), 24 hr	GG genotype GA genotype AA genotype P = not significant	6925 (1855-15,345) pg/mL[e], n = 19 3792 (1783-14,130) pg/mL ,n = 10 7778 pg/mL, n = 1	25
Paediatric renal transplant recipients	PBMC, PHA (1 μg/mL) or LPS (2 μg/mL), 18 hr	GA/AA genotype GG genotype $P = 0.001$, not significant[h]	600 ± 100, 4200 ± 200 ng/mL[g, h, i] 200 ± 25, 3700 ± 100 ng/mL n (total) = 8	22
Healthy volunteers	PBMC, Con A (5 μg/mL), 48 hr or anti-CD3 + -CD28 coated microspheres, 72 hr	GA/AA genotype GG genotype P = not significant, 0.586[k, l]	1350 ± 400[g, i, j], 3774 ± 666 pg/mL[k], n= 8 850 ± 250, 4099 ± 457 pg/mL, n = 24	26

continued Table 1:
[a] All incubations were carried out in 37°C, 5% CO_2.
[b] Data are given as means ± standard deviation.
[c] Data are given as median and interquartile range (range between brackets).
[d] Results are given after stimulation with respectively 10 and 1000 ng/mL endotoxin.
[e] Data are given as median and range (range between brackets).
[f] Results are given after stimulation with respectively 1 ng/mL of LPS for 24 hours or 100 ng/mL LPS for 3 hours.
[g] Data deduced from figures.
[h] Results are given after stimulation with respectively PHA and LPS.
[i] Data are given as means ± SEM.
[j] n (total) = 21 for Con A stimulation.
[k] Results are given after stimulation with respectively Con A and anti-CD3 + anti-CD28 coated beads.
[l] One sided *P* values

It may be argued that PBMCs are not the optimal cell types to test for TNF-α production because macrophages and monocytes are the main source of TNF-α producing cells (28). However, two other studies that tested *in vitro* TNF-α production by isolated monocytes found no significant association between the TNF-A –308 polymorphism and the level of *in vitro* production (29, 30). It should be mentioned that Pociot *et al.* did find a trend toward higher TNF-α production in TNF-A –308 GA-typed healthy volunteers (29), although they held the possible involvement of specific HLA-DR haplotypes rather than differential TNF-A –308 genotypes responsible. HLA-DR is close to the TNF-encoding gene and has been shown to be related to TNF-secretion (28, 31).
The contradictory results for the *in vitro* cell culture system of whole blood samples (20 versus 16, 23) may in part be explained by the difference in time period that endotoxin was allowed to stimulate; perhaps this time period was too short to allow optimal interaction among the various cell types to discriminate stimulated TNF-α production based on the TNF-A –308 genotypes of the tested individuals (28, 32).
The different stimulation protocols and the different TNF-α producing cell sources make it very difficult to compare whether patients and healthy individuals show differential or similar relationships regarding *in vitro* TNF-α production and TNF-A –308 polymorphism. In our study, no such relationship was found for liver transplant recipients or healthy volunteers. Neither in heart transplant recipients (16), nor multiple sclerosis patients (27), nor sarcoidosis patients (25) was a relationship established between *in vitro* TNF-α production and the TNF-A –308 polymorphism. In contrast, the studies involving paediatric renal transplant recipients or a mixed population of IBD patients and healthy volunteers did show a significant association (22, 21), although it was not clear from the latter study how patients and healthy volunteers were distributed within that population.
In summary, we cannot deduce a uniform *in vitro* TNF-α production profile based on the TNF-A –308 polymorphism, either in patients or in healthy individuals.

Only one other study has reported on the relationship between TNF-d3 gene polymorphism and *in vitro* TNF-α production (Table 2). There, TNF-d3 positive heart transplant recipients were found to produce significantly more TNF-α than TNF-d3 negative patients, using a cell culture system involving whole blood and endotoxin stimulation (16). From our study and the study by Turner *et al.* (16), it seems that TNF-d3 +ve individuals are likely to produce more TNF-α than TNF-d3 –ve individuals. Evidently, other studies are needed to confirm these findings.

Table 2. Association between *in vitro* TNF-α production and TNF-d3 cytokine gene polymorphism

Study population	Stimulation protocol[a]	Genotype	Results	Ref.
Heart transplant recipients	Whole blood, endotoxin (5 μg/mL), 4 hr	d3-positive d3-negative $P = 0.02$	616 (322-672) pg/mL[b], n = 13 241 (130-306) pg/mL, n = 9	16

[a] All incubations were carried out in 37°C, 5% CO_2.

[b] Data are given as median and interquartile range (range between brackets).

Studies that tested the relationship of IFN-G +874 gene polymorphism to *in vitro* IFN-γ production are reviewed in Table 3. The IFN-G +874 T allele was also referred to as allele number two, in the IFN-γ encoding gene (33). In these studies, a cell culture system using PBMC was applied.

The IFN-G +874 T-allele is generally claimed to be associated with high *in vitro* production of IFN-γ (3, 13). The study be Pravica *et al.* (34) describes a significant association between higher *in vitro* IFN-γ production by IFN-G +874 TT-genotyped healthy individuals than in –AT/AA positive individuals. The results of our study and those of Hoffmann *et al.* (26) show a tendency toward a similar association, using a stimulation protocol with Con A rather identical to that in Pravica's study (34). Other *in vitro* stimulation protocols either confirmed (anti-CD3 + anti-CD28 coated beads, LPS) or denied (anti-CD2, PHA) the relationship between high *in vitro* IFN-γ production and IFN-G +874 T allele in healthy individuals (22, 26, 35-37). In paediatric renal transplant recipients, the IFN-G +874 TT genotype was confirmed to be related to higher IFN-γ production than in AA-genotyped patients (22), whereas in our study for liver transplant recipients, no significant associations between *in vitro* IFN-γ production and the IFN-G +874 polymorphism were found.

In summary, evidence suggests that the relationship between high *in vitro* IFN-γ production and the IFN-G +874 allele is plausible for healthy individuals. For patients, the situation is not clear. The heterogeneity of the observed association between *in vitro* IFN-γ production and the IFN-G +874 polymorphism may be attributable to the fact that different patient populations were tested: liver transplant recipients in our study, paediatric renal transplant recipients (22), and adult renal transplant recipients (37). Also, in our study and the abovementioned studies, different *in vitro* stimulation protocols were used: respectively, Con A (in our study), PHA/LPS (22), and a completely different *in vitro* allostimulation culture system (37).

Table 3. Association between *in vitro* IFN-γ production and IFN-G +874 cytokine gene polymorphism[a]

Study population	Stimulation protocol[b]	Genotype	Results	Ref.
Healthy volunteers	PBMC, Con A (5 μg/mL), 48 hr	2/2 genotype 2/x genotype x/x genotype *P* = 0.01	425 ± 225 pg/mL[c, d], n = 8 225 ± 125 pg/mL, n = 14 125 ± 175 pg/mL, n = 8	34
Healthy volunteers	PBMC, PHA (1 μg/mL), 48 hr	12/12 genotype 12/x genotype x/x genotype *P* = not significant	4349 ± 2109 pg/mL[e], n = 4 3601 ± 1557 pg/mL, n = 7 1872 ± 816 pg/mL, n = 9	36
Healthy volunteers	PBMC, PHA (1 μg/mL), 48 hr	2/2 genotype 2/3 genotype 3/3 genotype *P* = 0.41	275 (214-2301) pg/mL[f], n = 3 531 (92-8000) pg/mL, n = 10 1241 (61-4438) pg/mL, n = 9	37
Paediatric renal transplant recipients	PBMC, PHA (1 μg/mL) or LPS (2 μg/mL), 18 hr	TT genotype AA genotype *P* = 0.006, 0.003[g]	30 ± 5, 17 ± 1 ng/mL[c, e, g] 3 ± 2, 3 ± 2 g/mL n (total) = 8	22
Renal transplant recipients	PBMC cryopreserved, pretransplantation, mixed lymphocyte culture antidonor spleen, 96hr	2/2 genotype 2/3 genotype 3/3 genotype *P* = 0.75	720 (0-4311) pg.mL[f], n = 8 1546 (0-3606) pg/mL, n = 22 374 (9-3804) pg/mL, n = 9	37
Healthy volunteers	PBMC cryopreserved, 2x anti-CD2 (50 ng/mL each), 48 hr	2/2 genotype 2/3 genotype 3/3 genotype *P* = not significant	Data not given N (total) = 111	35
Healthy volunteers	PBMC, Con A (5 μg/mL), 48 hr or anti-CD3 + -CD28 coated microspheres, 72 hr	TT genotype TA genotype AA genotype *P* = 0.107, 0.005[i, j]	2200 ± 550[c, e, h], 46,019 ± 3546 pg/mL[i], n = 5 2450 ± 500, 31,939 ± 3483 pg/mL, n =19 900 ± 250, 31,461 ± 6675 pg/mL, n = 6	26

[a] All incubations were carried out in 37°C, 5% CO_2.
[b] Data are given as means ± standard deviation.
[c] Data are given as median and interquartile range (range between brackets).
[d] Results are given after stimulation with respectively 10 and 1000 ng/mL endotoxin.
[e] Data are given as median and range (range between brackets).
[f] Results are given after stimulation with respectively 1 ng/mL of LPS for 24 hours or 100 ng/mL LPS for 3 hours.
[g] Data deduced from figures.
[h] Results are given after stimulation with respectively PHA and LPS.
[i] Data are given as means ± SEM.
[j] n (total) = 21 for Con A stimulation.

Previously published studies in which associations were investigated between *in vitro* IL-10 production and IL-10 –1082 gene polymorphism are shown in Table 4. The IL-10 –1082 GG and –AG genotypes are generally termed high-producing and the IL-10 –1082 AA genotypes the low-producing genotypes (3, 13). In reporter gene assays, the IL-10 –1082*G promoter was found to be more active than the IL-10 –1082*A promoter (38). Turner *et al.* (39) found the IL-10 –1082 GG genotype significantly associated with high *in vitro* IL-10 production. A similar study using

stimulation with Con A found similar results with a very small number of individuals tested, and *P* values were not calculated (40); however, these results were not confirmed in another study using a smaller amount of Con A for stimulation (26). Stimulation protocols with either LPS (22) or anti-CD3 + anti-CD28 coated beads (26) found the IL-10 –1082 GG genotype significantly associated with high *in vitro* IL-10 production. When PHA- or anti-CD2 *in vitro* stimuli were applied, no significant relationships between *in vitro* IL-10 production and the IL-10 –1082 polymorphism were established (22, 35, 37).

Table 4. Association between *in vitro* IL-10 production and IL-10 -1082 cytokine gene polymorphism

Study population	Stimulation protocol[a]	Genotype	Results	Ref.
Healthy volunteers	PBMC, Con A (10 μg/mL), 48 hr	GG genotype AG/AA genotype *P* = 0.035, correlation independent of polymorphisms at –819/-592	1720 ± 184 pg/mL[b], n = 12 1297 ± 101 pg/mL, n = 25	39
Healthy volunteers	PBMC, Con A (10 μg/mL), 48 hr	GCC/GCC[c]; GCC/ACC, GCC/ATA; ATA/ATA, ACC/ATA, ACC/ACC; No *P* value mentioned	749 pg/mL, n = 1 457 pg/mL[d], n = 6 334 pg/mL. n = 3	40
Healthy volunteers	PBMC, PHA (1 μg/mL), 48 hr	GG genotype AG genotype AA genotype *P* = 0.50	608 (70-828) pg/mL[e], n = 8 226 (35-2886) pg/mL, n = 14 181 (9-1992) pg/mL, n = 4	37
Paediatric renal transplant recipients	PBMC, PHA (1 μg/mL) or LPS (2 μg/mL), 18 hr	GG genotype AA genotype *P* = not significant, 0.029[f]	0.15 ± 0.05, 0.95 ± 0.05 ng/mL[b, f, g] 0.05 ± 0.01, 0.40 ± 0.10 ng/mL n (total) = 8	22
Renal transplant recipients	PBMC cryopreserved, pretransplantation, mixed lymphocyte culture antidonor spleen, 72hr	GG genotype AG genotype AA genotype *P* = 0.50	10 (0-142) pg/mL[e], n = 17 13 (0-184) pg/mL, n = 24 8 (1-57) pg/mL, n = 8	37
Healthy volunteers	PBMC cryopreserved, 2x anti-CD2 (50 ng/mL each), 48 hr	GG genotype AG genotype AA genotype *P* = not significant[h]	Data not shown n (total) = 111	35
Healthy volunteers	PBMC, Con A (5 μg/mL), 48 hr or anti-CD3 + -CD28 coated microspheres, 72 hr	GCC/GCC[c]; GCC/ACC, GCC/ATA; ATA/ATA, ACC/ATA, ACC/ACC; *P* = not significant, 0.002[i, k]	550 ± 200[b, g, i], 5832 ± 853 pg/mL[k], n = 5 450 ± 100, 2702 ± 400 pg/mL, n = 13 450 ± 100, 2086 ± 415 pg/mL, n = 12	26

[a] All incubations were carried out in 37°C, 5% CO_2.
[b] Data are given as means ± SEM.
[c] Given are the combinations of gene polymorphisms for the IL-10 encoding gene-promoter at the positions –1082, -819, and –592, respectively.

[d] Data are given as means.
[e] Data are given as median and range (range between brackets).
[f] Results are given after stimulation with respectively PHA and LPS.
[g] Data deduced from figures.
[h] For the polymorphism at –819 C/T, no significant association with *in vitro* IL-10 production was found.
[i] n (total) = 21 for Con A stimulation
[j] Results are given after stimulation with respectively Con A and anti-CD3 + anti-CD28 coated beads.
[k] One-sided *P* values

In contrast to the abovementioned observations, which either did or did not confirm the presumed relationship between high *in vitro* IL-10 production and the IL-10 –1082 GG genotype, in our study we found completely opposite results. Our study showed a significant association between the GG genotype and low *in vitro* IL-10 production. Because in our patient population, as well as in our healthy control population, the distribution of the presumed high, intermediate, and low IL-10 producing haplotypes –respectively, GCC/GCC (high), GCC/ACC, GCC/ATA (intermediate), and ATA/ATA, ACC/ATA, ACC/ACC (low) (41)- was exactly identical to the IL-10 –1082 GG versus –GA versus –AA genotypes, we also found the GCC/GCC haplotype associated with low *in vitro* IL-10 production. We have no explanation for the different findings between our study and that of Turner *et al.* (39), because in both studies Con A was used as the stimulating agent and healthy volunteers were involved. Summarizing all findings, we cannot deduce a uniform relationship between the level of *in vitro* IL-10 production and the IL-10 –1082 polymorphism in healthy individuals.

For comparison of the IL-10 *in vitro* production/IL-10 polymorphism relationships between patients and healthy individuals, our analyses of the reviewed literature were based on the same studies as for the IFN-γ associations. Similarly as for the IFN-γ studies, the heterogeneity of associations between *in vitro* IL-10 production and the IL-10 –1082 genotypes may be attributable to the diversity of patient populations such as liver transplant recipients (our study), paediatric renal transplant recipients (22), and adult renal transplant recipients (37); and the diversity of *in vitro* stimulation protocols, respectively Con A (in our study), PHA/LPS (22), and a completely different *in vitro* allostimulation culture system (37).

Only one other research group reported on the association between *in vitro* production of IL-13 and gene polymorphism of the IL-13 encoding gene (Table 5). They found that *in vitro* IL-13 production by IL-13 –1055 TT individuals was significantly harder to suppress by the *in vitro* stimulation protocol than in CT/CC-genotyped individuals (42). These results were obtained in a mixed population of allergic asthma patients and healthy volunteers, and similar results were also obtained in the separated patient population.

In our data, we found no confirmation for this observation, because neither in healthy volunteers nor in liver transplant recipients we found a significant association

Table 5. Association between *in vitro* IL-13 production and IL-13 -1055 cytokine gene polymorphism[a]

Study population	Stimulation protocol[b]	Genotype	Results	Ref.
Allergic asthma patients and nonatopic healthy volunteers	PBMC, anti-CD28 (5 μg/mL) and PMA (10 ng/mL) and anti-CD2 (1 μg/mL), 3 days	TT genotype	Reduced inhibition IL-13 production	42
		CT genotype	Almost complete inhibition IL-13 production	
		CC genotype	Almost complete inhibition IL-13 production	
		$P = 0.0016$ (TT versus CT), $P = 0.0002$ (TT versus CC), $P = 0.003$ (patients TT versus patients CT/CC)	Exact n not given	

[a] In literature, no reports associating IL-13 +2043 cytokine gene polymorphism with *in vitro* IL-13 production have yet been published.
[b] All incubations were carried out in 37°C, 5% CO_2.

between *in vitro* IL-13 production and the IL-13 –1055 gene polymorphism. Very few patients and healthy volunteers in our study possessed the IL-13 –1055 TT genotype, which may have influenced the results.

For the IL-13 +2043 polymorphism, we did find a trend toward higher IL-13 production in GG-genotyped liver transplant recipients compared with –AG/AA patients, whereas in healthy volunteers no significant association was found. In literature, no data have been reported on *in vitro* IL-13 production and this particular polymorphism. Altogether, at this moment it seems premature to denote individuals with certain IL-13 gene polymorphisms as high or low IL-13 producers.

We would have liked to address the question of whether in patients the possible genetic contribution to levels of cytokine production might be overruled by more vigorous "environmental" stimuli (such as the disease itself, or the medication or immunosuppression), compared with "naive" healthy individuals in whom a genetic contribution to cytokine production might be more easily revealed. Unfortunately, this interesting question could not be properly addressed based on the currently published studies. More studies are required, conducted like that of Mycko *et al.* (27) and like our study, in which both healthy individuals and patients are tested by the same *in vitro* stimulation protocols. In favor of such an approach, an interesting observation was made: a relationship between IL-6 cytokine gene polymorphism and *in vitro* IL-6 production was established in "truly naive" neonates, whereas in healthy adults, no significant relationship could be shown (43).

This article clearly illustrates the weak consensus on the relationship between certain cytokine gene polymorphisms and *in vitro* cytokine production. We advocate a multicenter workshop in which a uniform *in vitro* stimulation protocol is applied to test such a relationship. Until then, we suggest that in studying the association of

diseases or disease manifestations with cytokine gene polymorphisms, these polymorphisms described by their genotypic localization.

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Part III

Animal model: *in vitro* cytokine production profiles, gene polymorphisms and liver allograft survival

Warlé MC, Metselaar HJ, Kusters JG, Zondervan PE, Hop WCJ, Segeren KCA, Kwekkeboom J, IJzermans JNM, Tilanus HW.
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Transplant International 2004; in press.

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Part III

Chapter 6

Strain-specific *in vitro* cytokine production profiles do not predict liver allograft survival

The aim of this study was to assess whether differences in cytokine production between inbred rat strains could explain differences in liver allograft survival. Splenocytes from five different strains were cultured with Concanavalin A in order to determine *in vitro* cytokine production profiles. Strain-specific TNF-α, IFN-γ, IL-6 and IL-10 responses in naive animals were not associated with survival after rat liver transplantation. To investigate whether *in vitro* cytokine responses changed during the allogeneic inflammatory response, Brown Norway livers were transplanted to Lewis and Pivold Virol Glaxo recipients. During the early postoperative course IL-6 and IL-10 (Th2-like) responses were significantly up-regulated in Lewis recipients, whereas Th2-like responses were not increased in Pivold Virol Glaxo.
In conclusion: our results do not support the generally held view that differential *in vitro* cytokine responses are related to liver allograft survival, but suggest that cytokine responses are affected by the allogeneic inflammatory response after liver allografting.

Introduction

Liver allografts can be spontaneously accepted in fully mismatched donor recipient combinations of rat inbred strains (1, 2). In major histocompatibility complex (MHC)-identical strains August and Pivold Virol Glaxo (AUG and PVG, both RT1^a), AUG reject PVG livers in delayed fashion while in the reverse direction (AUG into PVG) the grafts show long-term survival (3). This indicates that non-MHC genes in the recipient are important in determining the strength of rejection. Possible candidates of genes determining the strength of rejection and liver allograft survival may be found in a range of cytokine genes.

Since differential cytokine production may explain differences in the outcome after allografting, polymorphisms in cytokine genes and *in vitro* production of cytokines have been investigated. In the rat IL-4 gene, a promoter region polymorphism has been identified which was associated with the level of IL-4 production *in vitro* (4). In human studies, polymorphisms in several human cytokine genes have been related to higher or lower production of cytokines upon *in vitro* stimulation (5-8). Furthermore, some cytokine gene polymorphisms have been associated with the occurrence of human solid organ graft rejection (9-13). More recently, differential levels of *in vitro* TNF-α production have also been associated with human liver allograft rejection (14, 15). *In vitro* cytokine responses can be affected by factors such as age, underlying disease and immunosuppressive therapy (16-18). These factors may enhance or negate the relevance of cytokine production profiles with respect to allograft rejection. To avoid these possible confounding factors, we used an animal model to study strain-specific *in vitro* cytokine responses in relation to liver allograft survival. Zimmerman et al. (3) reported that inbred rat strains exhibit remarkable differences in survival after liver transplantation. For example, DA, LEW and PVG recipients spontaneously accept liver allografts across a complete allogeneic barrier without the need for treatment to promote acceptance, whereas BN and AUG reject liver allografts in a short-term manner. Differences in strain-specific cytokine production profiles may influence the severity of the allogeneic inflammatory response after liver allografting. Therefore, we hypothesize that strain-specific *in vitro* cytokine production profiles could predict survival after liver allografting in the rat. We determined the endogenous capacity of lymphocytes to produce Th1- (TNF-α, IFN-γ) and Th2- (IL-6, IL-10) type cytokines upon a standardized *in vitro* stimulus in five different naive rat inbred strains (AUG, BN, DA, LEW and PVG).

The generally held view is that cytokine production profiles determine the severity of the inflammatory response, although it is also possible that cytokine production in response to an allogeneic stimulus increases due to more severe inflammation. Therefore, we also tested the hypothesis that strain-specific *in vitro* cytokine production profiles are affected by the allogeneic inflammatory response after liver transplantation.

For this purpose, long-term surviving BN livers were allografted to recipient strains with respectively high (LEW) and low (PVG) cytokine responses in naive animals.

Materials and Methods

Animals

Pathogen-free male inbred AUG/OlaHsd (RT1^{a}), BN/RijHsd (RT1^{n}), DA/OlaHsd (RT1^{a}), LEW/HanHsd (RT1^{l}) and PVG/OlaHsd (RT1^{c}) rats were obtained from Harlan (The Netherlands). Animals of all strains weighed approximately 250 grams (12 weeks of age). They were kept under specific pathogen-free conditions and fed a standard pellet diet and water *ad libitum*. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the European Commission (1986). The "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands, approved the specific protocols.

Liver transplantation

Orthotopic liver transplantation was performed by means of the technique as described by Kamada and Calne (19), using the cuff technique without hepatic artery revascularization. In brief, the donor liver was flushed by the portal vein and hepatic artery with a hypotonic citrate buffer solution through a canulla placed in the aorta. After recipient hepatectomy the suprahepatic vena cava was anastomosed with a 8-0 vascular suture. The portal vein and infrahepatic vena cava cuffs were then secured with a free ligature. The bile duct was rejoined by end-to-end anastomosis over an internal stent. The portal vein was clamped for less than 20 minutes in all animals.

Eight LEW (RT1^{l}) and 8 PVG (RT1^{c}) rats were used as recipients of BN (RT1^{n}) liver grafts and were killed on postoperative day 7 and 21, four animals per time point. The transplanted livers were removed for histology along with the spleen for isolation and culture of lymphocytes. In addition, two animals of both recipient strains were killed on postoperative day 100 to confirm spontaneous long-term survival of BN liver allografts as described by others (3, 20).

Liver allograft survival

In Table 1 we summarized liver allograft survival as reported in literature (1, 3, 20-24) to assess a possible relationship between the capacity to produce cytokines upon *in vitro* stimulation and liver allograft survival without immunosuppression. When recipients of liver allografts survived for more than 100 days, they were classified as long-term survivors. Of the five different recipient strains summarized in Table 1,

long-term graft survival was observed in DA, LEW and PVG, but LEW only showed long-term graft survival when BN donors were used. AUG and BN displayed rejection of all liver allografts in short-term (median survival less than 30 days) or delayed fashion (more than 30 days).

Table 1. Liver allograft survival between different inbred rat strains

Recipient strain (MHC)	Donor strain	Liver graft survival	Ref.
AUG ($RT1^a$)	BN	Delayed	1
AUG ($RT1^a$)	DA	Short	1
AUG ($RT1^a$)	LEW	Short	1
AUG ($RT1^a$)	PVG	Delayed	1
BN ($RT1^n$)	AUG	Delayed	1
BN ($RT1^n$)	DA	Short	1
BN ($RT1^n$)	LEW	Delayed	1, 2
BN ($RT1^n$)	PVG	Delayed	1
DA ($RT1^a$)	AUG	Long	1
DA ($RT1^a$)	BN	Long	1
DA ($RT1^a$)	LEW	Long	1, 3, 4
DA ($RT1^a$)	PVG	Long	1
LEW ($RT1^l$)	AUG	Short	1
LEW ($RT1^l$)	BN	Long	1, 2, 7
LEW ($RT1^l$)	DA	Short	1, 4, 6
LEW ($RT1^l$)	PVG	Short	1, 5
PVG ($RT1^c$)	AUG	Long	1
PVG ($RT1^c$)	BN	Long	1, 7
PVG ($RT1^c$)	DA	Long	1, 2
PVG ($RT1^c$)	LEW	Delayed	1

References in Table 1:

1. Zimmerman et al. (3)
2. Kamada et al. (20)
3. Sun et al. (21)
4. Farges et al. (22)
5. Wang et al. (23)
6. Ogura et al. (24)
7. This study

Histopathology

Tissues from liver allografts obtained at postoperative day 7 and 21 were fixed in buffered formalin. Paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin. All sections were scored in a blinded fashion by a pathologist (PEZ) according to the Banff criteria for grading liver allograft rejection, including portal inflammation, bile duct inflammation or damage and venous endothelial inflammation (25).

Isolation of lymphocytes from spleen

Spleens from naive animals (AUG, BN, DA, LEW, PVG) were minced and mashed through a fine sieve in phosphate-buffered saline. Mononuclear leucocytes were isolated from red blood cells and polymorphonuclear leucocytes by centrifugation over a Ficoll gradient (Ficoll-Paque Research Grade, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cells collected from the interface were washed twice, taken up in RPMI 1640 medium (Dutch modification, Gibco, Life Technologies Ltd., Paisly, Scotland) supplemented with 3 mM L-glutamine (Gibco) and 10% fetal calf serum and stored overnight at 37°C in a humidified atmosphere containing 5% CO_2. Lymphocytes from spleens of LEW and PVG at postoperative day 7 and 21 were isolated according to the same protocol and frozen with 10% DMSO and 20% fetal calf serum in liquid nitrogen before culture.

In vitro cytokine production

After overnight storage in RPMI 1640 medium (Gibco) at 37°C or after thawing from liquid nitrogen, viable splenocytes from respectively naive (n=7) and allografted animals (n=8) were counted using trypan blue exclusion. Subsequently, splenocytes (mainly lymphocytes) were cultured in RPMI 1640 medium with L-glutamine and 10% fetal calf serum with 10 $\mu g\ ml^{-1}$ Concanavalin A (Con A, Sigma Biosciences St Louis, MO, USA) at 5 X $10^5\ ml^{-1}$ in 100 μl replicates in 96 well U-bottom plates (Costar tissue culture treated polystyrene, Corning Inc., Corning, NY, USA). Initially, five replicate cultures were set up in order to harvest culture supernatants at day 1, 2, 3, 4 and 7 after onset of cell culture. Furthermore, lymphocytes from naive animals (3 animals per strain) were cultured with Con A (10 $\mu g\ ml^{-1}$), bacterial lipopolysaccharide (10 $ng\ ml^{-1}$) and phytohemagglutinin (5 $\mu g\ ml^{-1}$) to evaluate the effect of different *in vitro* stimuli. Culture supernatants were stored at -20°C until cytokines were measured. After thawing culture supernatants, IL-6, IL-10, IFN-γ and TNF-α levels were determined using commercially available ELISA assays (Cytoscreen Immunoassay Kit, Biosource International Inc., Camarillo, California, USA) according to the manufacturer's instructions. Cytokine concentrations were calculated by reference to manufacturer supplied cytokine standards and expressed in $pg\ ml^{-1}$.

Flowcytometry

To assess a possible correlation between *in vitro* cytokine responses and the spleen cell composition of different inbred rat strains, the percentages of viable monocytes, B and T lymphocytes were determined by flowcytometry using different specific antibodies. Splenocytes (10^5 cells) were incubated (30 minutes, 4°C) with mouse monoclonal antibody ED1, recognizing monocytes and macrophages (26) and OX19, an anti-rat CD5 monoclonal antibody, exclusively expressed on T cells (Serotec,

Oxford, UK). Biotinylated rabbit anti-rat IgM/IgG polyclonal antibodies were used to identify B lymphocytes (The Jackson Laboratory, Bar Harbor, ME, USA). Antibody binding was detected using phycoerythrin conjugated rabbit anti-mouse IgG (Becton Dickinson, San Jose, CA, USA) or streptavidin-PE (Caltag, Burlingame, CA, USA). The percentage of positive cells was measured using FACScan flowcytometry (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded from analysis based on staining with 7-amino-actinomycin D viability probe (BD Pharmingen, San Diego, CA, USA). Isotype antibodies were included as controls.

Statistical analysis

Differences in mean cytokine (IL-6, IL-10, IFN-γ, TNF-α) production measured in supernatants at consecutive days after onset of cell culture, between different strains were analyzed by repeated measurements analysis of variance. For this analysis all cytokine levels were transformed logarithmically in order to obtain approximate normal distributions. The PROC MIXED program from the SAS-package (version 6.12) was used for analysis of variance. Pairwise comparisons between strains were only performed if the overall test was significant. The relationship between relative numbers of lymphocytes/monocytes and *in vitro* cytokine responses were evaluated by Spearman's rank correlation (SPSS version 10). All results are expressed as mean values ± standard error of the mean.

Results

***In vitro* cytokine production profiles of naive rat inbred strains**

Mean cytokine levels during the period of culture (1 to 7 days) in response to Con A stimulation were compared between naive animals from AUG, BN, DA, LEW and PVG strains (7 animals per strain). Analysis of variance showed that the mean level of TNF-α production during the culture period was significantly higher in DA as compared to other strains (Table 2). AUG had significantly lower levels of TNF-α production as compared to BN, LEW and PVG (intermediate TNF-α producers). Concerning the *in vitro* production of IFN-γ, AUG and PVG (low producers) had significantly lower levels than BN, LEW and DA (high producers). As compared to other strains, DA and LEW produced significantly higher levels of IL-6. PVG produced significantly lower levels of IL-10 as compared to BN, DA and LEW, whereas AUG showed intermediate production of IL-10. When the effect of different *in vitro* stimuli was evaluated, only DA showed higher TNF-α and IL-6 responses upon Con A stimulation and BN produced higher levels of IFN-γ in response to Con A as compared to LPS and PHA stimulation. There were no further differences in cytokine responses upon Con A, LPS or PHA stimulation (data not shown).

Table 2. Classification of inbred rat strains into (relative) high, intermediate or low cytokine producers (7 animals per strain).

Strain	TNF-α[a]	[TNF-α] day 3 (pg/ml)[b]	P	IFN-γ[a]	[IFN-γ] day 3 (pg/ml)[b]	P	IL-6[a]	[IL-6] day 3 (pg/ml)[b]	P	IL-10[a]	[IL-10] day 3 (pg/ml)[b]	P
AUG	LOW	14.1 ± 6.7	0.008	LOW	21.9 ± 8.6	1.000	LOW	60.9 ± 52.1	0.795	INT	49.1 ± 10	0.066
BN	INT	93.9 ± 21.6	0.566	HIGH	224 ± 79	0.050	LOW	37.9 ± 24.7	0.709	HIGH	277 ± 150	0.003
DA	HIGH	293 ± 38.1	0.005	HIGH	437 ± 200	0.006	HIGH	105 ± 50.6	0.013	HIGH	240 ± 157	0.001
LEW	INT	167 ± 42	0.417	HIGH	317 ± 154	0.077	HIGH	103 ± 21.5	0.017	HIGH	137 ± 30	0.017
PVG	INT	227 ± 114		LOW	39.9 ± 11.1		LOW	24.7 ± 9.7		LOW	32.2 ± 9.3	

[a] Mean TNF-α, IFN-γ, IL-6 or IL-10 production was classified as high or low when mean cytokine production levels differed significantly from strains with respectively lower or higher levels.

[b] To illustrate the classification into high, intermediate and low responders cytokine production levels at day 3 of cell culture are shown in this table. Cytokine production levels at day 2, 4 and 7 of culture showed the same picture, whereas at day 1 of cell culture differences between inbred strains were not yet manifest.

Composition of splenocytes

To assess whether differences in cytokine responses upon *in vitro* stimulation were influenced by the relative number of different cell types, splenocytes from naive animals were analyzed by flowcytometry. Figure 1 shows that there were only small differences in the relative number of monocytes, B and T lymphocytes isolated from spleens of different rat inbred strains. When percentages of monocytes, B and T cells were related to *in vitro* cytokine responses, there were no significant correlations with TNF-α, IFN-γ, IL-6 or IL-10 production.

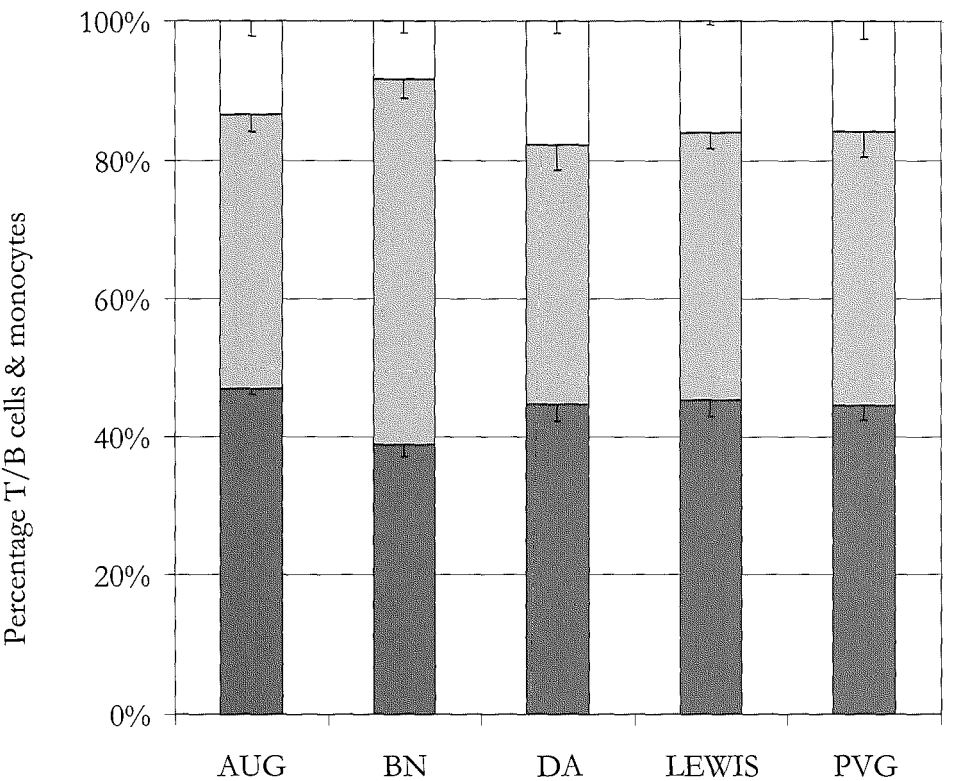

Figure 1. Relative number of monocytes (□), B (▒) and T (▓) lymphocytes isolated from spleens of naive animals (7 animals per strain) of different rat inbred strains.

In vitro cytokine production profiles after liver allografting

To investigate whether *in vitro* cytokine production profiles of high (LEW) and low (PVG) responder strains changed during the early course after liver transplantation, we used LEW and PVG recipients since both strains accept BN livers despite

differences in naive cytokine production profiles. When sections of liver allograft tissue were scored according to Banff criteria for grading liver allograft rejection, all animals showed moderate to severe acute rejection (Banff score 5 to 9) at postoperative day 7 and 21 and there were no significant differences in rejection grade between LEW and PVG (Table 3).

Table 3. Banff grading of liver allografts obtained at postoperative day 7 and 21 from LEW and PVG recipients of BN liver allografts

	LEW	PVG	*P* value[a]
Day 7	6, 6, 8, 9 [7.25]	6, 6, 7, 9 [7.0]	1.000
Day 21	5, 6, 6, 8 [6.25]	7, 8, 8, 8 [7.75]	0.143
Day 100	1, 2 [1.5]	2, 2 [2]	-

[a] *P* values from Mann-Whitney test.

In accordance with *in vitro* TNF-α production by naive animals, LEW and PVG did not differ in TNF-α levels when production was tested at postoperative day 7 and 21 (Figure 2a). For the Th1-type cytokine IFN-γ, production was significantly up-regulated at postoperative day 21 as compared to day 7 ($P \leq 0.02$) in both strains. Although mean postoperative IFN-γ levels (day 21) were higher in LEW than PVG, statistical significance was not reached ($P=0.16$) (Figure 2b). Concerning the Th2-type cytokines IL-6 and IL-10, higher production of IL-10 at postoperative day 21 by the LEW strain did not reach statistical significance ($P=0.13$). When cytokine production was compared between time-points after liver transplantation, LEW showed significant up-regulation of IL-6 and IL-10 at postoperative day 21 (respectively, $P<0.01$ and $P=0.03$), whereas IL-6 and IL-10 were not significantly increased in PVG (Figure 2c and d). Post-transplant cytokine responses in allografted animals were determined using nitrogen stored splenocytes, resulting in slightly lower responses as compared to cytokine production levels of freshly isolated splenocytes from naive animals (data not shown). The lowered response (due to freezing) precludes the comparison of post-transplant cytokine responses with pre-transplant cytokine production levels. In summary, although PVG showed low to intermediate cytokine responses in naive animals, we found a small but significant up-regulation of the IFN-γ response from postoperative day 7 to 21. In LEW we found up-regulation of both Th1- (IFN-γ) and Th2-type (IL-6 and IL-10) cytokine responses upon *in vitro* stimulation during the early course after liver transplantation.

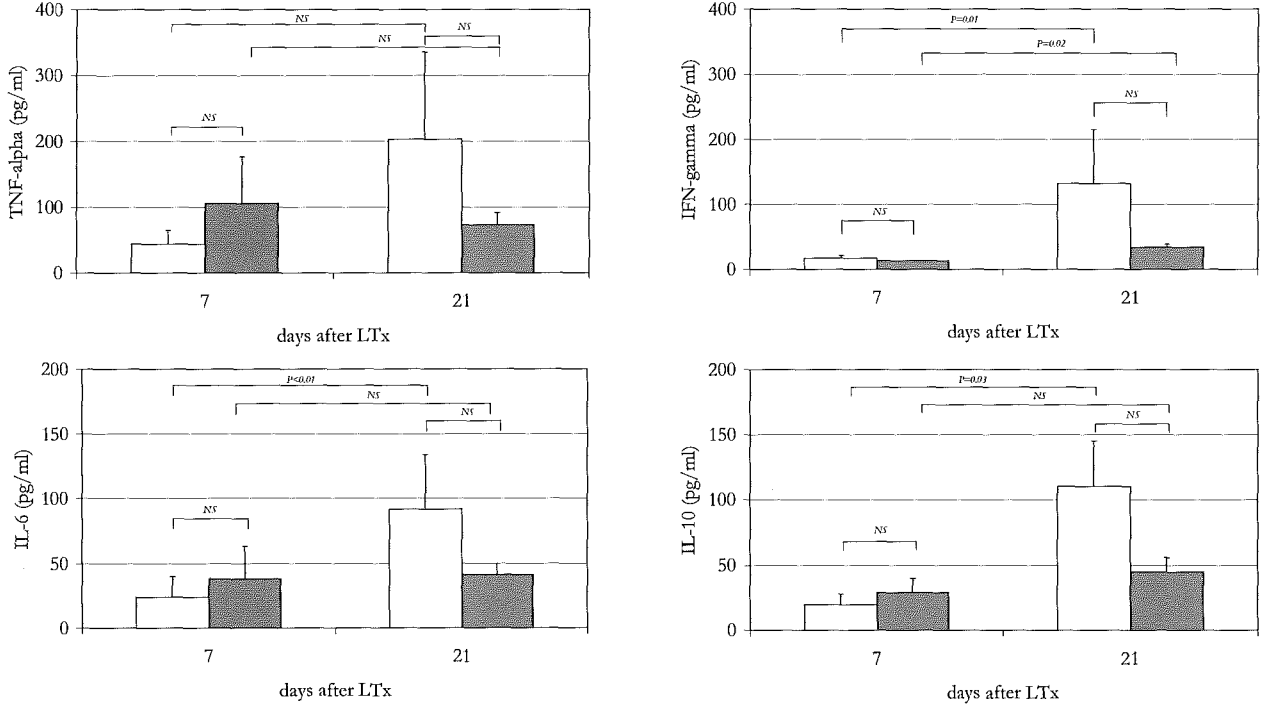

Figure 2. Post-transplant *in vitro* cytokine reponses in LEWIS (□) and PVG (▨) recipients of BN liver grafts. Mean cytokine production after 3 days of culture with a standardized Concanavalin A stimulus is shown in this figure. (A) LEW and PVG did not show a significant up-regulation of TNF-α levels from postoperative day 7 to 21. (B) IFN-γ production was significantly up-regulated at postoperative day 21 as compared to day 7 ($P \leq 0.02$) in both strains. (C) LEW showed significant up-regulation of IL-6 at postoperative day 21 ($P<0.01$), whereas IL-6 was not significantly increased in PVG. (D) LEW showed significant up-regulation of the IL-10 response at postoperative day 21 as compared to day 7 ($P=0.03$).

Discussion

Results from this study show that naive strain-specific *in vitro* cytokine production profiles do not predict allograft survival after rat liver transplantation. For example, DA and PVG, both showing long-term graft survival, produced respectively high and low levels of IFN-γ, IL-6 and IL-10. Furthermore, AUG and BN, both rejecting liver allografts in short-term or delayed fashion, produced respectively lower and higher levels of TNF-α, IFN-γ and IL-10. This observation does not confirm the role of cytokine production profiles in naive animals as promoters of the immune response after liver allografting. A possible explanation for this finding is that naive cytokine responses are affected by the allogeneic response during the course after liver transplantation. To test this hypothesis post-transplant cytokine responses were studied in high (LEW) and low (PVG) responder strains. When BN donors were used, both strains showed spontaneous long-term survival and there were also no significant differences in the strength of rejection during the early postoperative course (Table 3). In LEW recipients of BN allografts, both Th1- (IFN-γ) and Th2-type (IL-6 and IL-10) cytokine responses were significantly up-regulated during the early postoperative course, whereas Th2-type responses were not increased in PVG with lower Th1-like responses than LEW (Figure 2). These data indicate that the endogenous capacity of mononuclear cells to produce cytokines upon *in vitro* stimulation is modulated during the immune response after liver transplantation. The consequence of this finding is that pre-transplant cytokine responses not necessarily reflect the level of cytokine production after liver allografting.

Kamada *et al.* described that spontaneous liver allograft acceptance is not due to the absence of an immune response, there is clear histological evidence of acute rejection with mononuclear cell infiltration of portal tracts and sinusoids (27). In this study we also found histological evidence of moderate to severe rejection in LEW and PVG recipients of long-term surviving BN liver allografts. Moreover, a paradoxical early immune activation during the development of spontaneous liver allograft acceptance has recently been described (28) with an early up-regulation of pro-inflammatory cytokines in spleen and lymph nodes of the recipient (29). In this study we cultured mononuclear cells isolated from spleens of LEW and PVG recipients of long-term surviving liver allografts. Therefore, we speculate that increased IFN-γ responses as observed in LEW and PVG liver allograft recipients reflect a higher activation-state of the Th1-type cell population.

To our knowledge, there have been no previous reports on cytokine production upon *in vitro* stimulation in different inbred rat strains. We evaluated whether these strain-specific *in vitro* cytokine production profiles were influenced by the type of *in vitro* stimulus or variation in the composition of cell types used for culture. For AUG, LEW and PVG, cytokine production profiles upon stimulation with Con A,

LPS and PHA were comparable, whereas DA and BN seemed to produce higher levels of respectively TNF-α, IL-6 and IFN-γ upon stimulation with Con A (data not shown). We observed some variation in the composition of cell types isolated from spleens of different inbred strains (Figure 1), however the relative number of monocytes, B and T lymphocytes did not correlate with cytokine production levels. In our view these data suggest that differences in cytokine production profiles between inbred rat strains are, at least in part, a consequence of strain-specific factors regulating the activity of cytokine genes.

In accordance with results from this animal study, human studies investigating pre- and post-transplant *in vitro* cytokine responses did not find an association between differential production of IL-10 and the occurrence of acute liver graft rejection (14, 15). Since LEW and PVG did not differ in rejection grade and *in vitro* TNF-α responses, our results neither support nor contradict previous studies showing an association between higher *in vitro* production of TNF-α and the occurrence of acute human liver graft rejection (14, 15).

In conclusion, different inbred rat strains could be classified as high-, intermediate- or low-responders with regard to *in vitro* TNF-α, IFN-γ, IL-6 and IL-10 production since naive animals of different strains show differences in their capacity to produce cytokines upon a standardized *in vitro* stimulus. Although, cytokine gene polymorphisms associated with higher or lower production of cytokines, have been related to acute rejection of heart, kidney and liver transplants (9-13), our results indicate that strain-specific cytokine production profiles do not predict rat liver allograft survival. This finding does not support the generally held view that higher or lower *in vitro* cytokine responses determine the severity of allogeneic inflammatory responses and survival after liver allografting. An important question that has to be addressed, is whether *in vitro* cytokine responses are a cause or a consequence of the allogeneic inflammatory response after liver allografting. This study provides an argument in favour of the latter, since *in vitro* cytokine production profiles were found to be altered during the allogeneic response after rat liver transplantation.

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Chapter 7

TNF-α gene polymorphism and production among inbred rat strains with short- and long-term liver allograft survival

Differences in spontaneous allograft acceptance after liver transplantation among inbred rat strains might be explained by variation in the local production of TNF-α as a potent mediator of the inflammatory response. In this study we hypothesize that nucleotide differences in the rat *Tnf* gene influence TNF-α protein expression. As such, polymorphisms in the *Tnf* gene may also provide a possible explanation for differences in survival of allogeneic liver grafts among inbred rat strains. We therefore investigated the capacity of mononuclear cells to produce TNF-α in response to a mitogenic stimulus and the *Tnf* locus was sequenced in six different inbred rat strains. Among the six strains (AUG, BN, DA, LEW, PVG and WF) 44 nucleotide differences including 36 single nucleotide polymorphisms (SNPs), five simple sequence length polymorphisms, two deletions and one insertion, were found in the *Tnf* gene. Although, the inbred rat strains differed significantly in mean levels of maximum TNF-α production (P=0.001), no associations were found with nucleotide differences within the *Tnf* gene. In conclusion, our results indicate that differential *in vitro* TNF-α responses among inbred rat strains are not associated with nucleotide differences within non-coding regulatory regions within the rat TNF-α gene. Without an established relationship between polymorphisms and expression of the TNF-α gene, it is preliminary to address a possible association of *Tnf* gene polymorphisms with rat liver allograft survival.

Introduction

Inbred rat strains exhibit remarkable differences in spontaneous allograft acceptance after liver transplantation. For example, Dark Agouti (DA, $RT1^a$), Pivold Virol Glaxo (PVG, $RT1^c$), and Wistar Firth ($RT1^u$) recipients spontaneously accept liver allografts across a complete allogeneic barrier without the need for treatment to promote acceptance, whereas Brown Norway (BN, $RT1^n$) and August ($RT1^c$) reject liver allografts in a short-term manner (1, 2). Spontaneous acceptance of a transplanted liver is not due to the absence of an immune response, as there is clear histological evidence of acute rejection, with mononuclear cell infiltration of portal tracts and sinusoids (3, 4). Moreover, Bishop *et al.* (5, 6) reported that spontaneous liver allograft acceptance in rats is associated with early immune activation and expression of pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α in lymphoid tissues. TNF-α is, with interleukin-1 (IL-1), one of the proximal mediators of the inflammatory response (7). As such, it has extremely potent pro-inflammatory activities and it has been implicated in the pathogenesis of different immune-mediated diseases including acute rejection after solid organ transplantation.

In a previous study we showed that inbred rat strains with variable graft survival times after liver transplantation display significant heterogeneity in cytokine responses upon *in vitro* stimulation (4). Differences in TNF-α protein production levels among different inbred rat strains may be explained by altered regulation of TNF-α gene activity. TNF-α gene expression and its functional activity is regulated at different stages: gene transcription, post-transcriptional control of mRNA stability (8), the expression of TNF receptors (9) and stabilization by soluble TNF receptors (10). The rat gene for TNF-α is located within the class III region of the MHC which is a highly polymorphic region, and also the TNF-α gene itself contains a large number of polymorphisms (11). Although none of these polymorphisms have been shown to change the amino acid sequence of the TNF-α protein, many were found in non-coding regulatory regions and may thus influence expression levels.

The aim of this study is to examine whether differences in TNF-α responses upon *in vitro* stimulation among inbred rat strains with short- and long-term liver graft survival are associated with polymorphisms in the rat *Tnf* gene. Therefore the *Tnf* locus was sequenced in six different inbred rat strains with variable liver graft survival times (1, 2).

Materials and methods

Animals

Pathogen-free male inbred AUG/OlaHsd ($RT1^c$), BN/RijHsd ($RT1^n$), DA/OlaHsd ($RT1^a$), LEW/HanHsd ($RT1^l$), PVG/OlaHsd ($RT1^c$) and WF/HanHsd ($RT1^u$) rats

were obtained from Harlan (The Netherlands). Animals of all strains weighed approximately 250 grams (12 weeks of age). They were kept under specific pathogen-free conditions and fed a standard pellet diet and water *ad libitum*. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the European Commission (1986). The "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands, approved the specific protocols.

Isolation of splenocytes

Spleens from naive animals (AUG, BN, DA, LEW, PVG and WF) were minced and mashed through a fine sieve in phosphate-buffered saline. Mononuclear leukocytes were isolated from red blood cells and polymorphonuclear leukocytes by centrifugation over a Ficoll gradient (Ficoll-Paque Research Grade, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Cells collected from the interface were washed twice, resuspended in RPMI 1640 medium (Dutch modification, Gibco, Life Technologies Ltd., Paisly, Scotland) supplemented with 3 mM L-glutamine (Gibco) and 10% fetal calf serum and stored overnight at 37°C in a humidified atmosphere containing 5% CO_2.

Capacity of splenocytes to produce TNF-α in vitro

Viable splenocytes (predominantly lymphocytes) were counted using trypan blue exclusion. Subsequently, lymphocytes from 7 animals per strain were cultured in RPMI 1640 medium with L-glutamine and 10% fetal calf serum with 10 μg ml^{-1} Concanavalin A (Con A, Sigma Biosciences St Louis, MO, USA) at 5 X 10^5 ml^{-1} in 100 μl replicates in 96 well U-bottom plates (Costar tissue culture treated polystyrene, Corning Inc., Corning, NY, USA). Initially, four replicate cultures were set up in order to harvest culture supernatants at day 1, 2, 3, and 4 after onset of cell culture. Culture supernatants were stored at -20°C until cytokines were measured. After thawing culture supernatants, TNF-α levels were determined using commercially available ELISA assays (Cytoscreen Immunoassay Kit, Biosource International Inc., Camarillo, California, USA) according to the manufacturer's instructions. Cytokine concentrations were calculated by reference to manufacturer supplied cytokine standards and expressed in pg ml^{-1}.

Tnf gene PCR and sequence analysis

From all strains DNA was isolated from splenocytes (according to a standard protocol) and DNA was stored at -20°C. Target DNA amplification (PCR) was performed in a 50μL reaction volume containing 1.5 mmol $MgCl_2$, 200 mmol dNTP, 1 Unit Taq polymerase (PCR core system, Promega, USA) and 2 pmol of

each primer. The primers used are shown in table 1. Because the *Tnf* gene is approximately 3500 bp long, the DNA was amplified in three overlapping fragments. The PCR amplification consisted of 35 cycles. The cycles consisted of a 30-sec denaturation step at 95°C, a 3-min annealing step at 60°C and a 3-min extension step at 70°C. The final cycle was a 6-min extension step at 70°C to allow full product extension. Each experiment included negative contamination controls where template DNA was replaced by H_2O. All amplified bands of the anticipated sizes were considered a positive result. DNA sequence determination was performed directly on these PCR products, and sequences were analysed with the SECentral computer program (Scientific & Educational Software, Durham, NC, USA). The TNF-α DNA nucleotide sequences from AUG (GenBank accession number AY427673), PVG (AY427674) and WF (AY427675) have been deposited on the GenBank sequence database.

Table 1. Primers for rat *Tnf* genomic DNA amplification[a] and sequence analysis.

Primer name	*Primer oligonucleotide (5'- 3')*	*Position in the Tnf gene*[b]
	Forward	
F1	GGCTGAGTTCATTCCCTCTG	10
F2	GCTGGTGGGCACAGTAATG	1081
F3	ACGTGCAGAGATGTGCAGAG	1192
F4	GGTGAGTCTGTCTACTAACC	1443
F5	CAGCAGATGGGCTGTACCTT	2116
F6	TCCAATGGGCTTTCGGAACT	2685
F7	AGCTGTCTTCAGGCCAACAT	3004
	Reverse	
R1	GGGGAAAAGCTCTCATTCAA	588
R2	CGTGCTCATGGTGTCCTTTC	805
R3	TCTTGCTTCTTCCCTGTTCC	1242
R4	GTGGCCTCCTAATGCCTTG	1879
R5	AAATGGCAAATCGGCTGACG	2217
R6	GAACAGTCTGGGAAGCTCTGA	2853
R7	CTTTCCAGCAGGTATTTGG	3513

[a] Primer pairs used for DNA amplification were F1*R4 (1870 bp), F3*R5 (1026 bp), and F5*R7 (1398bp).
[b] The nucleotide numbering is from the BN strain sequence (GenBank accession number AF329984).

Statistical analysis

One way ANOVA was used to compare mean levels of maximum TNF-α production between different inbred rat strains (SPSS version 10). For analysis of the relation between *in vitro* TNF-α responses and single nucleotide polymorphisms within the *Tnf* gene among inbred rat strains, Mann-Whitney U test with Bonferroni correction for multiple comparisons was used. For the relationship between *in vitro* TNF-α responses and repeat length polymorphisms in the *Tnf* gene among inbred rat strains, SAS Proc Mixed linear regression analysis with 'rat strain' as random factor was used. $P \leq 0.05$ (two-tailed) was considered significant.

Results

Tnf gene nucleotide differences among different inbred rat strains

The *Tnf* gene was sequenced using genomic DNA from AUG, BN, DA, LEW, PVG and WF inbred rats. We identified 44 nucleotide differences (36 single nucleotide polymorphisms (SNPs), five simple sequence length polymorphisms, two deletions and one insertion) among these six strains. The polymorphisms were located in the promoter region, the introns, exon 4, the 3'-untranslated region (3'-UTR) and the 3' flanking region. The *Tnf* genomic DNA nucleotide sequence differences we observed among six rat strains are listed in Table 2. In the 5' promoter region we found 2 SNPs at nucleotides 298 and 395. Furthermore, we found a $(CA)_n$ repeat length polymorphism at nucleotide 344. Within the four *Tnf* gene exons, we only found polymorphisms in exon 4, at nucleotides 2030, 2180 and 2246. All three polymorphisms were due to nucleotide differences between BN and the other five strains. None of these polymorphisms changed the predicted amino acid sequence of the TNF-α protein. In the *Tnf* gene introns, we found 20 SNPs, a 17 base deletion (at position 1277), a 3 base deletion (at position 1434) and two $(G)_n$ length polymorphisms among the six strains. Only five of these polymorphisms were due to nucleotide differences between AUG, DA, LEW, PVG and WF (except BN). In the *Tnf* gene 3'UTR, we found seven SNPs, one $(C)_n$ length polymorphism and one insertion. Four additional SNPs and a $(C)_n$ length polymorphism were found in the 3' flanking region of the *Tnf* gene.

Differential in vitro TNF-α responses among inbred rat strains

For each animal the maximum level of cytokine production, measured during the period of cell culture, was determined at the day of maximum TNF-α production. Maximum TNF-α production was usually reached after two or three days of cell culture with Con A stimulation. Figure 1 shows differences in mean levels of maximum TNF-α responses among six inbred rat strains ($P=0.001$). After pairwise

Table 2. Polymorphisms of the *Tnf* gene among six inbred rat strains

Position in the Tnf gene[1]	*Polymorphism*	*Inbred rat strains*					
		AUG[2]	*BN*	*DA*	*LEW*	*PVG*[2]	*WF*[2]
Promoter							
298	SNP	A	G	G	G	A	A
334	$(CA)_n$ repeat	24	22	27	30	24	25
395	SNP	A	G	G	G	A	A
Intron 1							
985	SNP	G	G	A	A	G	G
1061	SNP	C	A	C	C	C	C
1115	SNP	T	C	C	C	T	T
1122	SNP	A	G	A	A	A	A
1166	SNP	T	GC	T	T	T	T
1187	SNP	T	C	T	T	T	T
1277	Deletion	-	X[3]	-	-	-	-
1359	SNP	G	A	G	G	G	G
1361	SNP	A	G	A	A	A	A
1400	SNP	T	G	T	T	T	T
1401	$(G)_n$ repeat	13	11	10	10	13	12
1422	SNP	T	G	T	T	T	T
Intron 2							
1434	Deletion	-	CTC	CTC	-	-	-
1640	SNP	T	G	T	T	T	T
1677	SNP	A	C	A	A	A	A
Intron 3							
1746	SNP	A	C	A	A	A	A
1755	$(G)_n$ repeat	8	11	10	9	8	8
1799	SNP	AC	CTC	AC	AC	AC	AC
1820	SNP	G	C	G	G	G	G
1847	SNP	G	C	G	G	G	G
1890	SNP	A	G	A	A	A	A
1904	SNP	A	G	A	A	A	A
1914	SNP	A	C	A	A	A	A
1922	SNP	A	G	G	A	A	A
Exon 4							
2030	SNP	A	G	A	A	A	A
2180	SNP	T	C	T	T	T	T
2246	SNP	C	T	C	C	C	C
3' UTR							
2436	SNP	A	G	A	A	A	A
2572	SNP	A	T	A	A	A	A
2630	SNP	T	C	T	T	T	T
2716	SNP	C	G	C	C	C	C
2771	SNP	T	C	T	T	T	T
2893	SNP	A	C	A	A	A	A
3080	SNP	C	T	C	C	C	C
3093	$(C)_n$ repeat	5	6	5	5	5	5
3297	Insertion	GTC	-	GTC	GTC	GTC	GTC
3'flanking region							
3353	SNP	A	G	A	A	A	A
3357	SNP	A	C	C	C	A	A
3375	$(C)_n$ repeat	3	6	3	3	3	3
3381	SNP	T	C	T	T	T	T
3392	SNP	T	C	T	T	T	T

[1] The nucleotide numbering is from the BN strain sequence (GenBank accession number AF329984).

[2] GenBank accession numbers: AY427673 (AUG), AY427674 (PVG) and AY427675 (WF).

[3] X = GAGATAAGGAGATATGA

comparison, significant differences were found between AUG *versus* DA ($P<0.001$), AUG *versus* LEW ($P=0.002$), AUG *versus* PVG ($P=0.003$), and AUG *versus* WF ($P<0.001$).

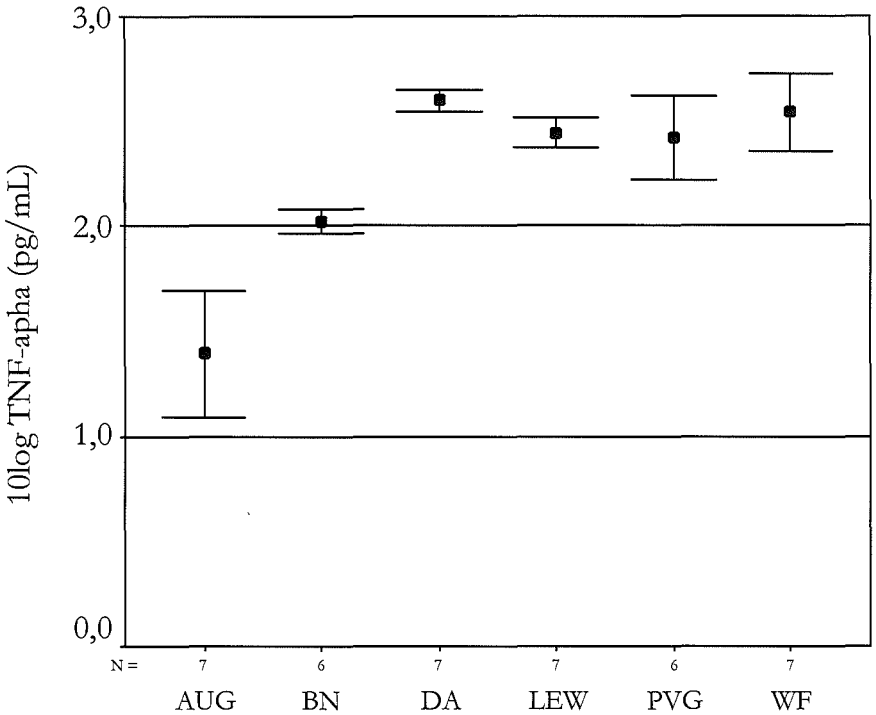

Figure 1. Mean levels of maximum TNF-α production of six inbred rat strains are shown and error bars represent standard error of the mean. ANOVA showed significant differences in maximum TNF-α production among the inbred rat strains ($P=0.001$). After pairwise comparison significant differences were found between AUG *versus* DA, AUG *versus* LEW, AUG *versus* PVG, and AUG *versus* WF ($P=0.003$).

In vitro TNF-α responses in relation to Tnf gene polymorphisms

To investigate whether nucleotide differences in the rat *Tnf* gene were associated with *in vitro* TNF-α responses, 36 SNPs, two deletions and one insertion among six inbred rat strains were related to maximum levels of TNF-α production. Thirty-three polymorphisms were differences in the nucleotide sequence of BN *versus* the other five strains; three (at position 298, 395 and 1115) between BN, DA, LEW *versus* AUG, PVG and WF; two (at position 1434 and 1922) between BN and DA *versus* AUG, LEW, PVG and WF; and one (at position 985) between DA and LEW *versus* AUG, BN, PVG and WF (Table 3). In total, four statistical comparisons were made and no significant associations were found.

Table 3. *In vitro* TNF-α production in relation to nucleotide differences in the TNF-α gene among six inbred rat strains.

Polymorphisms at positions	Mean ^{10}log TNF-α ± SD		*P*-value[a]
	Allele 1	Allele 2	
298, 395	G	A	
1115	C	T	
	2.37 ± 0.28	2.03 ± 0.84	1.000
985	G	A	
	2.03 ± 0.74	2.52 ± 0.17	0.088
1434	CTC	-[b]	
1922	G	A	
	2.33 ± 0.33	2.14 ± 0.75	1.000
1061	A	C	
1122, 1361, 1890, 1904, 2030, 2436, 3353	G	A	
1166	GC	T	
1187, 2180, 2630, 2771, 3381, 3392	C	T	
1277	GAGATAAGGAGATATGA	-[b]	
1359	A	G	
1400, 1422, 1640	G	T	
1677, 1746, 1914, 2893, 3357	C	A	
1799	CTC	AC	
1820, 1847	C	G	
2246, 3080	T	C	
2572	T	A	
2716	G	C	
3297	-	GTC[c]	
	2.02 ± 0.14	2.22 ± 0.70	0.348

[a] Mann-Whitney U, Fisher exact test with Bonferroni correction for multiple comparisons.
[b] deletion
[c] insertion

Linear regression analysis was used to assess the relationship between repeat length polymorphisms in the rat *Tnf* gene with maximum TNF-α production levels (Table 4). No significant associations were found between the $(CA)_n$ repeat length polymorphism at position 334, the $(G)_n$ repeat length polymorphisms at positions 1401 and 1755 and *in vitro* TNF-α responses.

Discussion

To our knowledge there have been no previous attempts to link *Tnf* gene nucleotide differences to TNF-α cytokine responses in rat strains. The data presented in this study indicate that polymorphisms in non-coding regulatory regions of the TNF-α gene are not associated with the capacity of mononuclear cells to produce TNF-α

Table 4. In vitro TNF-α production in relation to CA repeat length polymorphisms in the TNF-α gene among six inbred rat strains.

$(CA)_n$ or $(G)_n$ repeat polymorphism at position[b]	Correlation coefficient	*P*-value[a]
334	0.087	0.210
1401	-0.208	0.109
1755	0.070	0.692

[a] Linear regression analysis
[b] Linear regresion analysis could not be performed for the $(C)_n$ repeats at positions 3093 and 3375.

in response to a mitogenic stimulus. Of the 44 polymorphisms we identified among the six strains, 35 polymorphisms were differences between the BN strain and the other five strains. This observation indicates that the BN strain is phylogenetically more distantly related to the other five strains than they are to another, which is in accordance with a recently published phylogenetic study of rat inbred strains (12). In the 5' promoter region, we identified a $(AC)_n$ repeat length polymorphism at nucleotide 334 and two SNPs at nucleotides 298 and 395. Although, it has been suggested that microsatellite repeats within promoter regions of eukaryotic genomes are involved in regulation of gene expression with increased repeat length associated with increased gene transcription (13, 14), no significant association was found between the length of the $(AC)_n$ repeat and the level of maximum TNF-α production upon *in vitro* stimulation. This indicates that the $(AC)_n$ repeat length polymorphism does not influence TNF-α gene transcription, or alternatively that *in vitro* TNF-α responses are controlled by regulatory sequences outside the *Tnf* locus. Polymorphisms in the 3' UTR of the *Tnf* gene may as well be important with regard to the level of TNF-α protein production, since the 3' UTR has been reported to regulate the stability and translation of TNF mRNA in mice (15, 16). However, our analysis did not show a significant association of polymorphisms in the 3' untranslated region with *in vitro* TNF-α responses.

Differences in the composition of splenocytes used for cell culture and strain-specific variability in response to a certain stimulation protocol are non-genetic factors that might explain differences in TNF-α responses among inbred rat strains. However, in a previous study we showed that mononuclear cell populations isolated from spleens of inbred rat strains have a very similar composition (4). Furthermore, it has been shown that variability in cytokine production levels related to stimulation protocols was small as compared to interindividual differences in cytokine production profiles (4, 17). Therefore, we assume that differential *in vitro* TNF-α responses among inbred rat strains are, at least in part, controlled by genetic factors.

Between the MHC identical strains AUG and PVG, both $RT1^c$, no differences in the nucleotide sequence of the TNF-α gene were found. This observation is in

accordance with the fact that the rat gene for TNF-α is located within the MHC (class III region). Interestingly, AUG and PVG differed significantly in the maximum level of *in vitro* TNF-α production (Figure 1). This finding suggests that *in vitro* TNF-α responses by mononuclear cells isolated from rat spleens are regulated independent of genetic variation within the MHC including the *Tnf* locus. Without an established relationship between *Tnf* gene polymorphisms and TNF-α protein expression, it remains speculative to speak of associations between TNF-α gene polymorphisms and rat liver allograft survival. In summary, our results indicate that differential *in vitro* TNF-α responses among inbred rat strains are not associated with nucleotide differences in non-coding regulatory regions within the TNF-α gene.

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Part III

Part IV

Cytokine gene polymorphisms and acute human liver graft rejection

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Part IV

Chapter 8

Cytokine gene polymorphisms and acute human liver graft rejection

Interindividual differences exist in the capacity to produce cytokines. It has been reported that levels of in vitro cytokine production measured after stimulated cell culture are associated with polymorphisms in cytokine genes. Moreover, a correlation between heart, kidney, liver and lung graft rejection or survival with cytokine gene polymorphisms has been described. In the present study, we analysed the association of gene polymorphisms in T helper subtype 1 (Th1-), Th2-, and regulatory type cytokines with human liver allograft rejection. Patients who received a primary liver graft from 1992 onward and were seen at the transplant outpatient clinic since then were included on this study (n=89). Patients were HLA typed routinely. Biopsy-proven acute rejection occurred in 41 of 89 patients. After informed consent, blood was collected and DNA was obtained. Using amplification-refractory mutation system polymerase chain reaction, the following cytokine gene polymorphisms were determined: IL-2 +166, IL-2 -330, IL-15 +13689, IL-15 -80, TNF-A -308, TNF-d3, IFN-G +874 (Th1 type cytokines), IL-4 +33, IL-4 -590, IL-6 -174, IL-10 -592, IL-10 -819, IL-10 -1082, IL-13 +2043, IL-13 -1055 (Th2 type cytokines), TGF-B1 +869, and TGF-B1 +915 (regulatory type cytokines). Univariate analysis showed that polymorphisms of IL-10 -1082, TGF-B1 +869 and HLA-DR6 were significantly related to liver graft rejection. Multiple logistic regression analysis was used to assess which variables remained significantly predictive of acute rejection. Multivariate analysis showed that TGF-B1 +869 and HLA-DR6 were independently associated with the occurrence of acute rejection. These findings suggest a role for the regulatory type cytokine transforming growth factor-β1 in human liver graft rejection.

Introduction

After liver transplantation, patients are administered immunosuppressive drugs to prevent graft rejection. Immunosuppression is associated with numerous side effects, such as increased risks for infection, cancer, osteoporosis, hypertension-related disease, diabetes and kidney malfunction (1-4). Currently, in most centers, all patients are on similar immunosuppressive therapy. Alternatively, a patient specific immunosuppressive scheme based on patients' needs might be realized if the likelihood for rejection in each patient could be predicted. Polymorphisms of cytokine genes are determined from patient DNA. These samples can be obtained from the patient while on the waiting list for transplantation. If a particular cytokine gene polymorphism were associated with rejection or graft acceptance, immunosuppression might be administered in a patient-specific way after transplantation based on the cytokine genotype of the patient.

Cytokines are involved in graft rejection. Such T helper subtype (Th1-type) proinflammatory cytokines as interleukin-2 (IL-2), IL-15, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) are associated mainly with graft rejection, whereas such Th2-type cytokines as IL-4 have been associated with graft acceptance (5-12). Also, the Th2-type cytokine IL-10 has been associated with graft tolerance (13), although some studies have shown an effect in rejection (14-16) and others have shown no association (6, 17). Expression of the Th2-type cytokine IL-6 may be upregulated (18, 19), downregulated (6) or unchanged (20) with respect to graft rejection.

Th1-type cytokines may downregulate Th2-type cytokines and vice versa (21). The regulatory type cytokine transforming growth factor-β1 (TGF-β1), which may influence both Th1 and Th2 arms of the immune response (22), has been found by some to be upregulated in rejection (23), whereas others have established no such relationship (16, 19). The reported differential involvement of the same cytokines in graft rejection varies with either type of organ transplanted or the research group that reports the results. To date, studies of the association of cytokine gene polymorphisms and graft rejection have focused mainly on these mentioned cytokines of which serum or plasma levels or messenger RNA expression were found to correlate with the occurrence of rejection.

In liver transplantation, it is unclear how the contribution of the primary liver disease, immunosuppression, or the alloresponse itself combines with genetic predisposition to rejection. One could imagine that a genetic predisposition for rejection might lie in cytokines that initiate the rejection-cascade, as well as in effector cytokines. Because it is unknown which cytokines may function as initiator or effector cytokines in human liver graft rejection, we decided to investigate gene polymorphisms in a large array of Th1-, Th2-, or regulatory-type cytokines. The specific polymorphisms in

each cytokine gene that we studied and their relation with transplant outcome are listed in Table 1. The aim of the study is to determine whether an association exists between gene polymorphisms of the Th1-, Th2-, or regulatory-type cytokines and acute liver graft rejection.

Table 1. Positive associations between cytokine gene polymorphisms and transplant outcome

Polymorphism	Genotype	Association[a]
Th1 cytokine		
TNF-A -308	A+	(together with IL-10 –1082 AA genotype) Multiple rejections after heart transplantation (27)
	A+	Multiple rejections after kidney transplantation in case of HLA-DR mismatched grafts (28)
	A+	Steroid-resistant rejections after kidney transplantation (28)
	A+	Death from acute cellular rejection after heart transplantation (29)
	AA	Acute rejection after liver transplantation (30)
	AA/AG	Rejection after kidney transplantation (31)
IFN-G +874[b] (58)	T+	Fibrosis after lung transplantation (59)
	T+	Greater requirement for ATG therapy after acute kidney graft rejection (60)
Th2 cytokine		
IL-10 –1082	AA	(together with TNF-A –308+ phenotype) Multiple rejections after heart transplantation (27)
	GG	Multiple rejections after kidney transplantation in case of HLA-DR mismatched grafts (28)
Regulatory cytokine		
TGF-B +869[c]	C+	Renal failure after heart transplantation (53)
TGF-B +915[d]	G+	Lung allograft fibrosis (42)
	GG	Coronary vasculopathy after heart transplantation (54)

Note: until now, no significant relation with transplant outcome has been established for the following cytokine gene polymorphisms: Th1-cytokines: IL-2 (+166 and –330), IL-15 (+13689 and –80), TNF-d3; Th2-cytokines: IL-4 (+33 and –590), IL-6 (-174), IL-10 (-592 and –819), IL-13 (+2043 and –1055).
Abbreviation: ATG, antithymocyte globulin.
[a] Studies described reported on a significant ($P < 0.05$) association with the specified cytokine gene polymorphisms. Studies that did not find an association with the specified polymorphisms and transplant outcome are not listed in this table.
[b] IFN-G +874 T allele correlates with the presence of 12 CA repeats, also mentioned allele no. 2 (58).
[c] TGF-B +869 correlates with codon 10.
[d] TGF-B +915 correlates with codon 25.

Materials and Methods

Patients

In this study, all primary liver transplant recipients who underwent transplantation from 1992 onward and were seen at the transplant outpatient clinic between August 1998 and July 1999 were consecutively enrolled. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center Rotterdam (The Netherlands), and blood was collected after informed consent was obtained. The total group included 89 patients who were administered triple or double immunosuppressive therapy.

After liver transplantation, biopsies were performed after deterioration of liver function and/or worsening of the clinical situation of the patient. Biopsy specimens were analyzed according to criteria of Snover et al (24); if a score of 2 or greater was reached, the occurrence of acute rejection was defined and patients were treated with three consecutive intravenous daily dosis of 1,000 mg of prednisolone. In our patient group, rejection episodes usually took place within the first month after transplantation, whereas a few patients also experienced rejection at a later time. Therefore, we defined rejectors as patients who experienced biopsy-proven and prednisolone-treated rejection within the first month after liver transplantation (n=41), whereas those who did not experience rejection within the first month after liver transplantation were termed nonrejectors (n=48). Patients who experienced rejection usually had one or two rejection episodes within the first month after liver transplantation. In the setup of our study, we did not further segregate rejectors based on the severity or number of rejection episodes.
Initial immunosuppressive therapy included cyclosporine A (CsA), prednisone and azathioprine in 71 patients; tacrolimus (FK506) and prednisone in 11 patients and CsA and prednisone in 7 patients. Within 3 months after transplantation, all patients were on maintenance immunosuppressive therapy: CsA (target level, 100 to 200 ng/mL) or tacrolimus (target level, 5 to 10 ng/mL) and prednisone. Typing of patients and liver donors for HLA classes I and II was performed using complement-dependent cytotoxicity and DNA-based typing methods (Tissue Typing Reference Laboratory, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands). Nonrejectors and rejectors did not differ significantly with respect to sex, age, ethnicity, immunosuppressive therapy, primary liver disease, or presence of HLA-A, HLA-B or HLA-DR mismatches. Nonrejectors and rejectors typed similarly for HLA-DR1 to 5 and HLA-DR 7 to 10 (main groups), whereas patients with HLA-DR6 were found significantly more often among rejectors than non-rejectors ($P = 0.002$).

Cytokine genotyping

DNA was isolated from blood samples of patients who underwent transplantation in the Erasmus Medical Center Rotterdam by the classic salting-out method, involving proteinase K digestion and ethanol precipitation. DNA samples were coded and sent to Manchester to perform cytokine genotyping.
TNFd3 microsatellite typing was performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (25). Amplification-refractory mutation system polymerase chain reaction (PCR) methods were developed for the single-nucleotide substitutions at positions –330 and +166 in the IL-2 gene; -590 an d+33 in the IL-4 gene; -174 in the IL-6 gene; -592, -819 and –1082 in the IL-10 gene; -1055 and +2043 in the IL-13 gene; -80 and +13689 in the IL-15 gene;

-308 in the TNF-A gene; +874 in the IFN-G gene; and +869 (codon 10) and +915 (codon 25) in the TGF-B1 gene, as previously described (26).
Briefly, DNA was amplified in a 10 µL reaction and final concentrations of reagents were 1 × customized PCR master mix (Abgene, Epsom, UK), 5 µmol/L of specific primer mix (specific primer mix consists of 10 µmol/L of generic primer and 10 µmol/L of one of the two allele-specific primers), 0.5 µmol/L of internal control primer mix (internal control primers are used to check for successful PCR amplification; they amplify a human growth hormone sequence) and 25 to 100 ng of DNA. The protocol for the PTC-100 PCR machine (MJ Research, Waltham, MA, USA) was as follows: 95°C for 1 minute, followed by 95°C for 15 seconds, 65°C for 50 seconds, and 72°C for 40 seconds (10 cycles) and 95°C for 20 seconds, 59° for 50 seconds, and 72°C for 50 seconds (20 cycles). Amplified products were monitored by electophoresis on a 2% agarose gel containing ethidium bromide (0.5 mg/mL). Cytokine genotypings of coded samples were sent from Manchester to Erasmus Medical Center Rotterdam, where the relationship between cytokine gene polymorphisms and rejection after liver transplantation was evaluated.

Statistical analysis

Each polymorphism in each cytokine gene was univariately analyzed for differences in distribution between nonrejectors and rejectors by means of Fisher's exact test. When a particular genotype was present in fewer than 10 patients, the presence or absence of a chosen allele was analyzed instead of the genotype. For example, the genotype determined at position –308 in the TNF-A gene was distributed within the patient group as follows: homozygous AA present 3 patients, heterozygous GA present in 36 patients and homozygous GG present in 50 patients. Because the genotype AA was present in fewer than 10 patients, we compared the A+ and A- individuals. The pooled A-allele positive group includes patients with the genotype AA and GA and the pooled A-allele-negative group includes homozygous GG patients. In Table 2, univariate *P* are given for cytokine genotypes or allele-positive groups in which particular genotypes were present in fewer than 10 patients.
This study was performed as an explorative analysis of an array of Th1-, Th2- and regulatory-type cytokines to study whether previously described polymorphisms in these cytokine genes could be correlated to the occurrence of graft rejection after liver transplantation. To minimize the risk that a correlation between a particular cytokine gene polymorphism and rejection was established by chance because of the large number of variables tested, only variables significantly related to rejection after univariate analysis were subjected to multivariate analysis. For multivariate analysis of data, we used multiple logistic regression analysis. *P* of 0.5 (two-tailed) is considered the limit of significance.

Results

Single-nucleotide polymorphisms and microsatellite polymorphisms were investigated in genes of the Th1-, Th2-, and regulatory-type cytokines. In Table 2, the distribution of cytokine genotypes is shown in nonrejectors and rejectors. After univariate analysis of data, no significant differences were observed in the presence of genotypes of Th1-type cytokines IL-2, IL-15, TNF-α or IFN-γ between nonrejectors and rejectors. Of the Th2-type cytokines IL-4, IL-6, IL-10 and IL-13 that were tested, nucleotides at position –1082 in the IL-10 promotor were distributed differently in rejectors and nonrejectors ($P = 0.05$); the GG genotype was found mainly in rejectors, whereas the AA genotype was predominant in nonrejectors. For the regulatory-type cytokines TGF-β1, there was a significant difference ($P = 0.01$) in distribution of the genotype at position +869 (codon 10) between nonrejectors and rejectors. Rejectors most often showed the TT or CT genotype, whereas only a few rejectors possessed the CC genotype. We performed multiple regression analysis to assess whether polymorphisms that were univariately significant, IL-10-1082 and TGF-β1, remained independently significantly predictive for acute liver graft rejection. Because HLA-DR6 was related to acute liver graft rejection as well (see Table 3), we also included this variable in multivariate analysis. This analysis showed that TGF-B1+869 and HLA-DR6 were independently associated with acute liver graft rejection (Table 4). Patients with the TGF-B1+869-TT and –CT genotypes were more likely to experience rejection than patients with the CC genotype (61% and 49% versus 19%, respectively). Also 70% of patients heterozygous or homozygous for HLA-DR6 experienced rejection compared with 34% of patients who did not possess the HLA-DR6 allele.

Further investigation of such potential confounding variables as sex, age, race, type of immunosuppression, time of transplantation, primary liver disease, HLA-A, HLA-B, and HLA-DR mismatches and any (other) HLA-DR typing or cytokine gene polymorphisms were not found to affect results of multivariate analysis.

Discussion

This study investigated whether a genetic predisposition involving polymorphisms of Th1-, Th2-, or regulatory-type cytokine genes was associated with the development of rejection after human liver transplantation. Cytokine gene polymorphisms studied were chosen to include and extend cytokine gene polymorphisms previously shown to be related to graft rejection. In this study, we restricted ourselves to patient-specific polymorphisms; donor cytokine gene polymorphisms were not analyzed because donor material was not available to us.

Table 2. Univariate analysis of associations between Th1-, Th2-, and regulatory type cytokine gene polymorphisms and acute human liver graft rejection

	Cytokine genotype			P^a
Th1 polymorphism				
IL-2 +166	GG	GT	TT	0.60
Nonrejectors	14	28	6	
Rejectors	16	20	5	
IL-2 –330	GG	GT	TT	0.11
Nonrejectors	20	14	14	
Rejectors	12	8	21	
IL-15 –80	CC	CT	TT	0.29
Nonrejectors	29	14	5	(T+/T-)
Rejectors	20	19	2	
IL-15 +13689	AA	AT	TT	0.11
Nonrejectors	18	21	9	
Rejectors	7	23	11	
TNF-A –308	AA	GA	GG	0.83
Nonrejectors	2	20	26	(A+/A-)
Rejectors	1	16	24	
TNF-d3	x/x	3/x	3/3	0.26
Nonrejectors	17	20	9	
Rejectors	11	25	5	
IFN-G +874	AA	AT	TT	0.10
Nonrejectors	10	25	13	
Rejectors	17	14	10	
Th2 polymorphism				
IL-4 +33	CC	CT	TT	1.0
Nonrejectors	39	9	0	(T+/T-)
Rejectors	34	5	2	
IL-4 –590	CC	CT	TT	0.18
Nonrejectors	36	11	1	(T+/T-)
Rejectors	36	3	2	
IL-6 –174	CC	CG	GG	0.60
Nonrejectors	6	18	24	
Rejectors	8	16	17	
IL-10 –592	AA	CA	CC	0.39
Nonrejectors	4	17	27	(A+/A-)
Rejectors	2	12	27	
IL-10 –819	CC	CT	TT	0.39
Nonrejectors	27	17	4	(T+/T-)
Rejectors	27	12	2	
IL-10 –1082	GG	AG	AA	0.05
Nonrejectors	9	22	17	
Rejectors	14	21	6	
IL-13 –1055	CC	CT	TT	0.83
Nonrejectors	29	16	3	(T+/T-)
Rejectors	23	15	3	
IL-13 +2043	AA	AG	GG	1.0
Nonrejectors	0	18	30	(A+/A-)
Rejectors	5	10	26	
Regulatory polymorphism				
TGF-B +869	CC	CT	TT	0.01
Nonrejectors	17	19	12	
Rejectors	4	18	19	
TGF-B +915	GG	GC	CC	0.77
Nonrejectors	40	8	0	(C+/C-)
Rejectors	36	5	0	

[a] Univariate *P* are given for cytokine genotypes or pooled alleles in case particular genotypes were present in fewer than 10 patients (see Materials and Methods).

Table 3. Patient characteristics

	Nonrejectors (n = 48)	Rejectors (n = 41)
Sex (men/women)	20/28	22/19
Age (yr)	49 ± 12	47 ± 11
Race (Caucasoid/non-Causcasoid)	40/8	38/3
Immunosuppression		
CsA/Prednisone	5	2
FK506/Prednisone	8	3
CsA/prednisone/azathioprine	35	36
Primary liver disease		
Hepatitis B	8	2
Hepatitis C	2	7
Primary biliary cirrhosis	4	7
Primary sclerosing cholangitis	9	6
Alcohol	5	6
Acute hepatic failure	10	8
Other[a]	10	5
HLA-DR1 to HLA-DR10[b]		
HLA-DR6[c]	9 (19)	21 (51)
HLA mismatches[d]		
HLA-A 0,1/2	28/20	21/20
HLA-B 0,1/2	15/33	16/25
HLA-DR 0,1/2	25/23	15/26

Note: values expressed as number of patients, mean ± SD, or number (percent).
[a] Other primary liver diseases were cryptogenic cirrhosis (n = 5), Crigler-Najjar (n = 1), Wilson's disease (n = 3), insulinoma (n = 1), α1-antitrypin deficiency (n = 1), Budd-Chiari (n = 2), autoimmune hepatitis (n = 1), and cirrhosis secondary to hemochromatosis (n = 1).
[b] Distribution of patient HLA-DR1 through HLA-DR10 typings were not significantly different between rejectors and nonrejectors, with the exception of HLA-DR6.
[c] Nine nonrejectors were typed as HLA-DR6 (homozygous, n = 2; heterozygous, n = 7) versus 21 rejectors (1 homozygous, 20 heterozygous); this difference was significant, $P = 0.002$.
[d] HLA 0, 1, or 2 mismatches; HLA-A zero mismatches: 8 nonrejectors, 1 rejector; HLA-B, zero mismatches: 1 nonrejector, 3 rejectors; HLA-DR, zero mismatches: 2 nonrejectors and 3 rejectors.

Table 4. TGF-B +869 and HLA-DR6 polymorphism independently associate with acute liver graft rejection by multivariate analysis

Polymorphism	Rejection[a]	OR	95% CI OR	*P*[b]
TGF-B +869				
CC	4/21 (19)	1	-	-
CT	18/37 (49)	3.7[c]	1.0-14.2	0.053
TT	19/31 (61)	6.9[c]	1.7-27.6	0.006
HLA-DR6				
x/x	20/59 (34)	1	-	-
x/6 + 6/6	21/30 (70)	4.6	1.7-12.7	0.003

Abbreviations: OR, odds ratio; 95% CI OR, 95% confidence interval of odds ration.
[a] Number of patients with acute rejection compared with total number of patients with that particular genotype or phenotype (percentage).
[b] *P* for comparison with the reference category (OR = 1)
[c] Not significant different from each other ($P = 0.24$).

Our results show that none of the polymorphisms of the Th1-type cytokines IL-2, IL-15, TNF-α and IFN-γ tested correlated significantly with acute liver graft rejection. To date, studies of the relationship between Th1-type cytokine gene polymorphisms and graft rejection have shown a tendency toward the TNF-308-AA or –AG genotype being associated with liver, kidney or heart allograft rejection, although univariate statistical significance was not reached in all instances (17-31). Patients or healthy individuals with the AA genotype have been reported to produce more TNF-α than persons with the AG or GG genotype after in vitro stimulation protocols, although this could not be confirmed in every study (25, 32, 33).

In liver transplantation, a study by Bathgate *et al.* (30) found that the TNF-A gene at position –308 correlated with graft rejection, whereas Jonsson *et al.* (34) could not confirm this strong correlation. Bathgate *et al.* (30) found that patients with the AA genotype experienced rejection significantly more often than patients with the AG or GG genotype. In this study by Bathgate *et al.* (30), 13% of patients were TNF-A-308 AA positive, whereas in studies by Jonsson *et al.* (34) and us, 0% and 3% of patients were positive for the AA genotype, respectively. The small numbers of TNF-308-AA positive individuals may explain why the study of Jonsson *et al.* (34) and the present study found no association between this particular polymorphism and rejection. Apparently, cytokine genotype frequencies vary among populations. In all three studies, patients were mainly of Caucasoid origin; nevertheless, Scottish patients from the study of Bathgate *et al.* (30) differed in frequency of TNF-A-308 genotypes from the Australian (Jonsson *et al.*) and the mainly Dutch (our study) populations ($P < 0.05$).

Regarding polymorphisms in the Th2-type cytokines IL-4, IL-6, IL-10 and IL-13, after univariate analysis, we found a significant association between the single-nucleotide polymorphism at position –1082 in the IL-10 gene and liver graft rejection; 61% of patients with the GG genotype experienced rejection, whereas 74% of AA-positive patients remained free from rejection. A similar tendency, not reaching statistical significance, was found for IL-10-1082 genotype distribution and the occurrence of rejection after kidney and liver transplantation (28, 30). In heart transplant recipients, rejection was found mainly among IL-10-1082-AA-positive patients, although these data were not significant after univariate analysis (27). In smaller cohorts of heart, kidney and liver transplant recipients, no significant associations between IL-10 genotype and rejection were established (35-37).

Healthy individuals with the IL-10-1082-GG genotype or IL-10-592/-819/-1082 haplotype, in which the IL-10-1082 component was of the IL-10-1082-GG genotype, showed the greatest IL-10 production after in vitro stimulation, whereas IL-10-1082-GA and –AA showed intermediate and low production, respectively (38, 39). Conceptually, IL-10 has been termed a Th2-type cytokine because of its ability to downregulate Th1-type cytokines and its involvement in allograft tolerance in mainly

animal models (7, 13). However, our data and those of others show that patients with an IL-10 genotype corresponding to high IL-10 production are more susceptible to rejection, whereas the IL-10 genotype corresponding to low production is found mainly among nonrejectors (28). Perhaps the notion, derived mainly from animal studies, that IL-10 has a role in human allograft tolerance needs reevaluation. It is possible that IL-10 may increase rejection through its Th2-type action of promoting humoral responses because the involvement of alloantibodies in acute rejection cannot completely be excluded (40, 41).

TGF-β1 may function as a regulatory cytokine because it can influence both the Th1 and Th2 arms of the immune response. TGF-β1 may stimulate or inhibit proliferation of different cell types, depending on its cooperation with other growth factors present (22). In this study, polymorphism in the TGF-B1 gene corresponding to codon 10 was associated with acute human liver graft rejection after univariate and multivariate analysis. Patients with the TGF-B1+869 TT genotype were found mainly among rejectors (61%), whereas only 19% of rejectors were of the CC genotype. Currently, no clear picture has emerged from literature that describes which particular TGF-B1+869 genotype is correlated to high or low in vitro or in vivo cytokine production (42, 43), with the exception of studies by Yamada (44), who found that in Japanese healthy or osteoporotic individuals, the TGF-B1+869-CC genotype correlated with high TGF-β1 serum levels and the –TT genotype with low TGF-β1 serum levels.

Our results are contrary to those of Bathgate *et al.* (30) and Jonsson *et al.* (34), who found no correlation between TGF-B1 genotype and liver graft rejection. TGF-B1+869 genotype frequencies in their patients were not significantly different from ours. An alternative explanation might lie in the immunosuppression used. It has been suggested that CsA and FK506 (tacrolimus) may differentially influence TGF-β1 production, although published evidence shows that both CsA and FK506 similarly increase TGF-β1 levels in mouse spleen cells or isolated glomeruli obtained from renal graft biopsies (45-47). Unfortunately, an analysis of the relationship between the TGF-β1 polymorphism and liver graft rejection could not be performed because too few of our patients were administered FK506.

It has been known for some time that patients with the HLA-DR6 phenotype have worse kidney graft survival and a trend toward lower liver graft survival (48-50). In the present study, we confirm that HLA-DR6-positive individuals are more susceptible to rejection than those of other HLA-DR genotypes. The HLA-DR gene lies on chromosome 6 in close proximity to the TNF-A gene (32). We analyzed whether patients positive for HLA-DR6 possessed TNF-A genotypes or expressed TNFd3 microsatellite patterns different from patients with another HLA-DR type. However, we could establish no such relation, making it unlikely that the HLA-DR6 effect is brought about by linkage to the TNF cytokine gene. A purely speculative explanation,

not based on present experimental evidence, might be that HLA-DR6 is a good presenter of allopeptides, allowing peptides to bind that give rise to significantly greater cellular responses (51).

In our patient population, indications for liver transplantation were diverse. Because of the heterogeneity within primary liver diseases, our study design did not allow for a case-control study in which rejectors and nonrejectors with, e.g. similar primary liver diseases, immunosuppressive therapy or age are pair-wise compared. Instead, we included only variables significantly related to acute liver graft rejection after univariate analysis into a multiple logistic regression analysis. This was done to minimize the risk that the correlation between a particular polymorphism (e.g. the variable IFN-G+874) and rejection was established by chance. After multivariate analysis, the polymorphism in the TGF-B1 gene at position +869 and the presence of the HLA-DR6 allele remained significantly associated with the occurrence of rejection after liver transplantation. Thus, we could establish a relationship between a genetic characteristic and susceptibility to rejection after liver transplantation. This does not necessarily mean that TGF-β1 itself remains the only cytokine responsible for graft rejection. Because TGF-β1 can influence both Th1 and Th2 arms of the immune response, it is highly likely that the rejection reaction is set into motion through the induction of particular cytokine patterns.

Alternatively, it is possible that TGF-β1 itself contributes to the rejection reaction. Studies describing the presence of TGF-β1 during chronic rejection of liver, heart and lung grafts have attributed the involvement of TGF-β1 to its repair capacities in promoting extracellular matrix formation leading to fibrosis (42, 52-54). Also, increased TGF-β1 messenger RNA levels have been found in (late) acute rejection biopsy specimens obtained during acute rejection (23, 55). Perhaps TGF-β1 itself is directly involved in acute rejection through its role in apoptosis. TGF-β1 can induce apoptosis of hepatocytes in vitro by reduction of antioxidant enzyme levels and uncreasing peroxide production in the cell (56). Moreover, in a liver transplant model in the rat, TGF-β1 expression correlated with hepatocyte apoptosis and allograft rejection (57). For future studies, we consider it worthwhile to investigate whether TGF-β1 cytokine gene polymorphisms may provide a rationale for individualized immunosuppression in liver transplant recipients.

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Part IV

Chapter 9

Cytokine gene polymorphisms and acute liver graft rejection: a meta-analysis

In the field of liver transplantation, seven reports have been published investigating the association between polymorphisms in cytokine genes and the occurrence of acute rejection in liver graft recipients. However, most individual studies lack the statistical power to detect a small to moderate effect of cytokine gene polymorphisms on the acute rejection rate. To overcome this problem, we performed a quantitative meta-analysis of seven gene association studies which were comparable with regard to definition of acute rejection and the type of immunosuppression used. In the overall analysis the IL-10 polymorphism at position –1082 was identified as a genetic risk factor for acute liver graft rejection; liver transplant recipients carrying the IL-10 –1082.A allele displayed a lower rejection rate (common OR 0.6, 0.4-0.9 95% CI). For the TNF-A –308 polymorphism, a common odds-ratio could not be calculated due to significant heterogeneity of odds-ratios between the studies (mean OR 1.4, 0.8-2.6 95% CI). No associations were found between acute liver graft rejection and SNPs in the IL-6 (position -174) and TGF-β1 (positions +869 and +915) genes. In conclusion, results from this meta-analysis suggest a role for the IL-10 – 1082 polymorphism in human liver graft rejection.

Introduction

As a consequence of the improved short-term survival due to refinement of surgical techniques and better post-operative management, long-term outcome of patients who have had a liver transplant is becoming the main concern for clinicians dealing with the long-term adverse effects of immunosuppressant drugs. During long-term follow-up, chronic rejection is currently responsible only for a small proportion of graft loss and some deaths, whereas immunosuppression-related complications as infections, cardiovascular disease, renal failure and de novo malignancy account for most of the cases (1). Therefore, safe reduction of immunosuppressive drugs tailored to specific patient's needs is crucial in improving long-term outcome. The ability to safely reduce, or in some cases completely withdraw, potentially toxic medications has been documented previously (2) but remains unpredictable.

In transplant medicine, the paradigm of cytokine gene polymorphisms influencing the expression of cytokines, and thus the severity of the allogeneic inflammatory response, has become quite popular. As potential predictors of the inflammatory response, cytokine gene polymorphisms might be useful to characterize clinically 'tolerant' patients (defined as those who require minimal or no immunosuppression after transplantation and who maintain normal long-term allograft function). Cytokine genotyping may further allow the prospective earlier withdrawal of immunosuppressive agents in order to minimize toxicity, especially in patients identified to be at low risk for rejection.

In the field of liver transplantation seven comparable association studies have been performed, investigating frequencies of cytokine gene polymorphisms in rejectors versus non-rejectors (3-9). However, most individual studies lack the statistical power to detect small or moderate gene effects due to their relatively small sample sizes. In this paper we present a quantitative meta-analysis of association studies on cytokine gene polymorphisms and acute liver graft rejection.

Materials and Methods

Selection and comparison of studies

We searched Pubmed for studies investigating cytokine gene polymorphisms as risk factors for acute rejection after human liver transplantation. Between April 2000 and March 2004 seven studies have been published on this subject (3-9). Table 1 shows that these seven studies are comparable with regard to definition of acute rejection and the type of immunosuppression used. All studies defined rejection as biopsy-proven episodes of acute rejection during the early post-transplant period, treated with high-dose steroids. Only Jonsson *et al.* (4) also included 10 patients with

Table 1. Comparison of studies

Ref.	Transplant center	Period	Patient characteristics (male/female, recipient age, ethnic origin)	Definition of acute rejection	Initial immunosuppression	Maintenance immunosuppression
3	Royal Infirmary of Edinburgh, United Kingdom	1992 – 1998	Not described	Liver biospy and treatment with high-dose steroids	CsA or tacrolimus, prednisolone (20 mg), azathioprine (1-2 mg/kg)	CsA or tacrolimus, prednisolone (5mg), azathioprine
4	University of Queensland, Brisbane, Australia	1988 – 1999	M/F: 70/51, median age: not described, ethnic origin: 121 white (not specified)	Liver biospy and treatment with high-dose steroids (AR within and after first 4 weeks)	CsA or tacrolimus, prednisone (3 to 0.3 mg/kg by day 7), with or without azathioprine (1 mg/kg)	CsA (300 ng/mL) or tacrolimus (10 ng/mL), prednisone
5	Rabin MC, Tel-Aviv, Israel	Not described	M/F: 32/36, age range: 20-69 yr, ethnic origin: 68 caucasian	Liver biospy (AR within first 6 weeks)	CsA, prednisone, azathioprine or tacrolimus, prednisone	CsA, prednisone, azathioprine or tacrolimus, prednisone
6	UMDNJ-New Jersey Medical School, Newark, NJ, USA	Not described	M/F: 26/27, median age: 57 yr, ethnic origin: 34 caucasian, 6 african american, 13 other	Liver biospy and treatment with high-dose steroids	Tacrolimus (10-15 ng/mL), prednisolone (200 to 20 mg by day 6)	Tacrolimus, prednisolone
7	Erasmus MC Rotterdam, The Netherlands	1992 – 2000	M/F: 42/47, median age: 48 yr, ethnic origin: 78 caucasian, 11 other	Liver biospy and treatment with high-dose steroids (AR within first 4 weeks)	CsA or tacrolimus, prednisone, with or without azathioprine	CsA (100-200 ng/mL) or tacrolimus (5-10 ng/mL), prednisone
8	Portland Veterans Administration MC, Portland, OR, USA	1989 – 1999	M/F: 156/54, age range: 18-69; ethnic origin: 187 white, 23 other	Liver biopsy and response to treatment with high-dose steroids (AR in 50% within 70 days)	CsA or tacrolimus, prednisone, azathioprine or MMF	CsA or tacrolimus, prednisone (10 mg), azathioprine or MMF
9	Virginia Commonwealth University, VA, USA	1999 – 2000	M/F: 44/33, age range: 24-60 yr, ethnic origin: 77 caucasian	Liver biospy (AR within first 8 weeks)	CsA or tacrolimus, steroids, MMF	CsA or tacrolimus, steroids, MMF

one or more 'late' episodes of acute rejection (> 4 weeks after liver transplantation) in the rejection group, whereas Jazrawi *et al.* (8) did not differentiate between 'early' and 'late' episodes of acute rejection. Immunosuppressive protocols in all studies consisted of a calcineurin inhibitor (cyclosporin or tacrolimus) and prednisone with or without azathioprine. MMF was only used in a subgroup of patients studied by Jazrawi *et al.* (8) and Mas *et al.* (9) Furthermore, all studies investigated adult recipients of liver allografts and the majority of these patients were Caucasians (not specified by Bathgate *et al.* and Jonsson *et al.*).

Statistics

The outcome measure was biopsy-proven acute rejection during the early post-transplant period. In the seven studies polymorphisms in IL-2, IL-15, TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-13 and TGF-β1 genes have been investigated. We performed a meta-analysis on five cytokine gene polymorphisms including TNF-A -308, IL-6 –174, IL-10 –1082, TGF-B1 +869 and TGF-B1 +915.

We used multiple logistic regression analysis to test for heterogeneity of odds-ratios between the studies (SPSS Version 10). If this analysis indicated that the odds ratios for acute rejection were not significantly different between the studies, we carried out an overall analysis and calculated a common odds-ratio with 95% confidence interval. In case of heterogeneity, a mean odds-ratio with 95% CI was calculated. All P values are two tailed and $P<0.05$ was considered significant.

Results

Association studies on cytokine gene polymorphisms and liver graft rejection

In seven studies, 16 different single nucleotide polymorphisms have been studied in Th1-, Th2- and regulatory-type cytokine genes including IL-2, IL-15, TNF-α, IFN-γ, IL-4, IL-6, IL-10, IL-13 and TGF-β1 (Table 2). Four polymorphisms in respectively, TNF-α, IL-10 and TGF-β1 cytokine genes were found to be associated with acute liver graft rejection. Bathgate *et al.*, Fernandes *et al.* and Mas *et al.* reported a significant association of a polymorphism at position –308 (A/G) in the promoter of the TNF-α gene with acute liver graft rejection, however this observation was not confirmed by 4 other studies (4, 5, 7, 8). Mas *et al.* also found a significant association with the TNF-α polymorphism at position –238 (A/G). Other significant associations were described by Warlé *et al.*: a promoter polymorphism at position –1082 (A/G) in the IL-10 gene and a SNP in the TGF-β1 gene at position +869 (T/C) were found to be associated with acute rejection after liver transplantation.

Polymorphisms in the IL-2, IL-15, IFN-γ, IL-4, IL-6 and IL-13 genes were only investigated in a few studies and no positive associations were found (3-9).

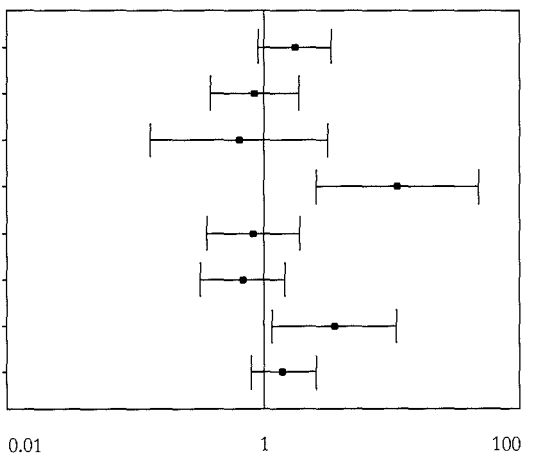
Table 2. Cytokine gene polymorphisms in liver transplant recipients in relation to acute rejection

Gene	Polymorphism	No.	Incidence of acute rejection (%)			Association	Ref.
Th1 type cytokines							
			TT	GT	GG		
IL-2	-330 T/G	89	21/35 (60)	8/22 (36)	12/32 (38)	No	7
			TT	TGT	GG		
IL-2	+166 T/G	89	5/11 (46)	20/48 (42)	16/30 (53)	No	7
			TT	CT	CC		
IL-15	-80 T/C	89	2/7 (29)	19/33 (58)	20/49 (41)	No	7
			TT	AT	AA		
IL-15	+13689 T/A	89	11/20 (55)	23/44 (52)	7/25 (28)	No	7
			AA	GA	GG		
TNF-α	-308 A/G	144	15/19 (79)	25/55 (46)	28/70 (40)	Yes	3
TNF-α	-308 A/G	121	0	14/49 (29)	23/72 (32)	No	4
TNF-α	-308 A/G	63	0	3/7 (43)	30/56 (54)	No	5
TNF-α	-308 A/G	53	1/1 (100)	7/12 (58)	5/40 (13)	Yes	6
TNF-α	-308 A/G	89	1/3 (33)	16/36 (44)	24/50 (48)	No	7
TNF-α	-308 A/G	210	2/10 (20)	9/58 (16)	31/142 (22)	No	8
TNF-α	-308 A/G	77	-	9/20 (45)	10/54 (19)	Yes	9
			GG	GA	AA		
TNF-α	-238 A/G	210	38/195 (20)	4/15 (27)	0	No	8
TNF-α	-238 A/G	77	7/51 (14)	12/23 (52)	-	Yes	9
			TT	AT	AA		
IFN-γ	+874 A/T	63	5/11 (46)	17/30 (57)	11/22 (50)	No	5
IFN-γ	+874 A/T	89	10/23 (44)	14/39 (36)	17/27 (63)	No	7
Th2 type cytokines							
			TT	CT	CC		
IL-4	-590 T/C	89	2/3 (67)	3/14 (21)	36/72 (50)	No	7
			TT	CT	CC		
IL-4	+33 T/C	89	2/2 (100)	5/14 (36)	34/63 (54)	No	7
			GG	CG	CC		
IL-6	-174 G/C	63	-	32/58 (55)	1/5 (20)	No	5
IL-6	-174 G/C	89	8/14 (57)	16/34 (47)	17/41 (42)	No	7
IL-6	-174 G/C	77	-	14/44 (32)	5/30 (17)	No	9
			AA	AG	GG		
IL-10	-1082 G/A	144	16/38 (42)	30/68 (44)	16/38 (42)	No	3
IL-10	-1082 G/A	121	7/28 (25)	16/60 (27)	14/33 (42)	No	4
IL-10	-1082 G/A	63	19/33 (58)	10/19 (53)	4/11 (36)	No	5
IL-10	-1082 G/A	53	4/19 (21)	7/27 (26)	2/7 (29)	No	6
IL-10	-1082 G/A	89	6/23 (26)	21/43 (49)	14/23 (61)	Yes	7
			AA	CA	CC		
IL-10	-592 C/A	121	-	12/43 (28)	25/78 (32)	No	4
IL-10	-592 C/A	89	2/6 (33)	12/29 (41)	27/54 (50)	No	7
			TT	CT	CC		
IL-13	-1055 T/C	89	3/6 (50)	15/31 (48)	23/52 (44)	No	7
			AA	AG	GG		
IL-13	+2043 A/G	89	5/5 (100)	10/28 (36)	26/56 (46)	No	7
Regulatory type cytokines							
			CC	CT	TT		
TGF-β1	+869 T/C	144	10/18 (56)	35/71 (49)	23/55 (42)	No	3
TGF-β1	+869 T/C	121	8/17 (47)	14/49 (29)	15/55 (27)	No	4
TGF-β1	+869 T/C	89	4/21 (19)	18/37 (49)	19/31 (61)	Yes	7
			CC	GC	GG		
TGF-β1	+915 G/C	144	3/4 (75)	9/20 (45)	56/120 (47)	No	3
TGF-β1	+915 G/C	121	0	6/16 (38)	31/105 (30)	No	4
TGF-β1	+915 G/C	89	0	5/13 (39)	36/76 (47)	No	7

Meta-analysis

Individual odds-ratios for the TNF-A –308.A allele are shown in Figure 1A. The test for heterogeneity of odds-ratios between studies showed that the association between the TNF-A –308.A allele and rejection differed significantly between the seven study-populations (*P*=0.007). Two studies gave significant associations with rejection, while the others did not. The mean odds-ratio was calculated to be 1.40 (95% CI 0.80-2.60) for the TNF-A –308.A allele. The common odds-ratio calculated for the IL-6 –174.C allele (0.59, 0.19-1.87 95% CI) indicates that there is no significant association with acute rejection (Figure 1B).

Studies	OR	95% CI	Sign.
Bathgate et al.	1.77	0.91 – 3.42	0.092
Jonsson et al.	0.85	0.39 – 1.88	0.693
Tambur et al.	0.65	0.13 – 3.18	0.595
Fernandes et al.	11.2	2.61 – 48.1	0.001
Warlé et al.	0.84	0.36 – 1.94	0.679
Jazrawi et al.	0.69	0.32 – 1.48	0.340
Mas et al.	3.60	1.18 – 10.9	0.025
Mean	1.40	0.80 – 2.60	-



Logistic regression analysis for heterogeneity between studies: df: 6, *P*=0.007.

Figure 1. (A) Logistic regression analysis of acute liver graft rejection in TNF-308 A positive versus TNF –308 A negative recipients.

Although all individual odds ratios of the IL-10 –1082.A allele as a rejection risk factor did not reach significance (Figure 1C), the common odds ratio (0.61; 0.39-0.94 95% CI) was statistically significant (*P*=0.027). This indicates that carriers of the IL-10 –1082.A allele had a lower acute rejection risk after receiving a liver allograft. The test for heterogeneity of odds-ratios between studies investigating the TGF-B1 +869.T allele was significant (*P*=0.006). Therefore, a common odds-ratio could not be calculated, whereas the mean odds-ratio was 0.90 (0.30-3.00 95% CI). The common odds-ratio of the TGF-B1 +915.C allele was 1.08 (0.59-1.96 95% CI). This indicates that the polymorphisms in the TGF-β gene were not associated with acute liver graft rejection (Figure 1D and 1E).

Studies	OR	95% CI	Sign.
Bathgate et al.	0.56	0.26 – 1.18	0.127
Jonsson et al.	0.48	0.21 – 1.11	0.086
Tambur et al.	2.21	0.58 – 8.47	0.249
Fernandes et al.	0.79	0.13 – 4.63	0.790
Warlé et al.	0.45	0.17 – 1.18	0.102
Common	0.61	0.39 – 0.94	0.027

Logistic regression analysis for heterogeneity between studies: df: 4, *P*=0.359.

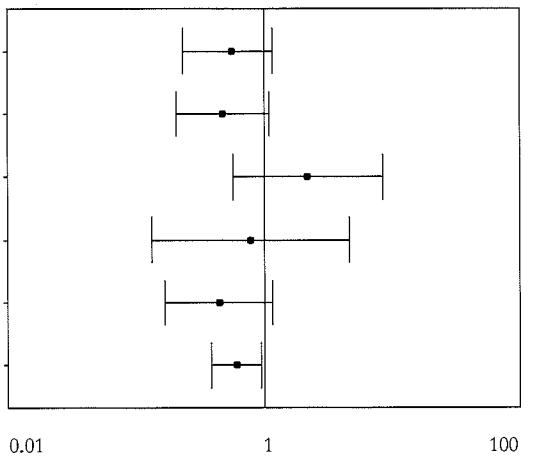


Figure 1. (B) Logistic regression analysis of acute liver graft rejection in IL-10 –1082 A positive versus IL-10 –1082 A negative recipients.

Studies	OR	95% CI	Sign.
Tambur et al.	4.92	0.52 – 46.7	0.165
Warlé et al.	0.59	0.19 – 1.86	0.368
Mas et al.	2.33	0.74 – 7.37	0.149
Common	1.46	0.70 – 3.03	0.313

Logistic regression analysis for heterogeneity between studies: df: 4, *P*=0.127.

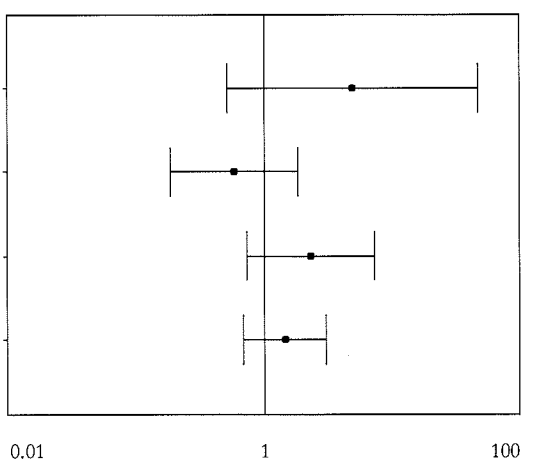
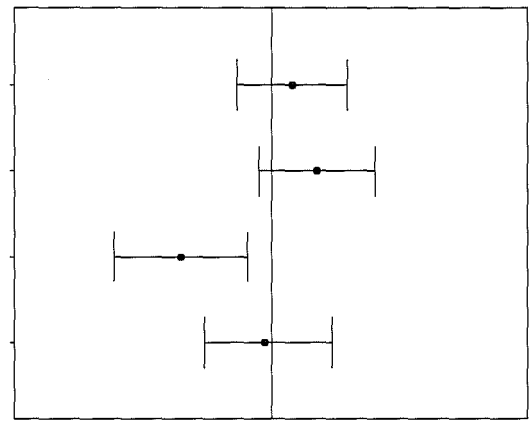


Figure 1. (C) Logistic regression analysis of acute liver graft rejection in IL-6 –174 G positive versus IL-6 –174 G negative recipients.

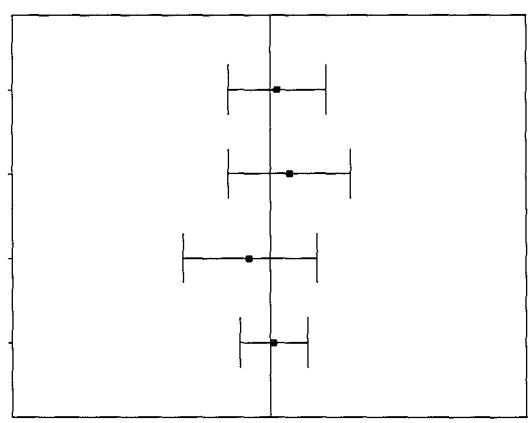
Studies	OR	95% CI	Sign.
Bathgate et al.	1.47	0.54 – 3.96	0.451
Jonsson et al.	2.29	0.81 – 6.53	0.118
Warlé et al.	0.20	0.06 – 0.65	0.007
Mean	0.90	0.390– 3.00	-



Logistic regression analysis for heterogeneity between studies: df: 2, P=0.006.

Figure 1. (D) Logistic regression analysis of acute liver graft rejection in TGF-B +869 T positive versus TGF-B +869 T negative recipients.

Studies	OR	95% CI	Sign.
Bathgate et al.	1.14	0.48 – 2.75	0.765
Jonsson et al.	1.43	0.48 – 4.28	0.520
Warlé et al.	0.69	0.21 – 2.32	0.553
Common	1.08	0.59 – 1.96	0.730



Logistic regression analysis for heterogeneity between studies: df: 2, P=0.675.

Figure 1. (E) Logistic regression analysis of acute liver graft rejection in TGF-B +915 C positive versus TGF-B +915 C negative recipients.

Discussion

Lack of statistical power to detect small or moderate gene effects is a major limitation for many gene association studies. Meta-analyses based on published data might be helpful to overcome this problem, however some concerns have to be addressed. Consistency in the definition of acute rejection as the main outcome measure, is the first condition that has to be fulfilled. In five studies acute rejection was defined as 'early' biopsy-proven acute rejection within the first 4 to 6 weeks after liver transplantation, treated with high-dose steroids. However, Jazrawi *et al.* (8) did not differentiate between 'early' and 'late' acute rejection episodes; in this study only 26% of all patients with acute rejection were diagnosed within the first postoperative month. An important argument for exclusion of 'late' acute rejections is that -in many cases- acute rejection after the early post-operative period (> 4-6 weeks) is the result of insufficient immunosuppression (e.g. due to non-compliance or tapering during infectious complications). Therefore, inclusion of 'late' acute rejections may bias the results of gene association studies. In the overall meta-analyses performed in this study, the number of patients with 'late' acute rejections included is small and therefore it is unlikely that the results are biased.

Differences in immunosuppressive regimens, i.e. type of induction therapy, the scheme of maintenance therapy and dosage of immunosuppressants, is also a potential source of bias. For example, cyclosporin increases the TGF-β1 production (10), thus the usage or dosage of cyclosporin might be more important than polymorphisms in the TGF-β1 gene. Furthermore, glucocorticoids that inhibit activation of NFκB, which is a transcription factor for many cytokine genes (11), play an important role in the production of certain cytokines and might overrule the effect of cytokine gene polymorphisms. All liver transplant patients included in this meta-analysis, received more or less the same type of immunosuppression: a calcineurin inhibitor and prednisone, with or without azathioprine. Nevertheless, there are some differences in dosages and target levels in blood as shown in Table 1. These 'minor' differences in immunosuppressive protocols provide a possible explanation for significant heterogeneity of odds-ratios between gene association studies investigating the TNF-A –308 and TGF-B1 +869 polymorphism (Figure 1A and 1D).

As previously described, ethnicity can strongly influence the distribution of cytokine gene polymorphisms (12). For example, Fernandes *et al.* observed a significant higher incidence of the TNF –308 AA genotype in the African American population when compared with the group that was predominantly white, implying a variation with ethnicity. However, ethnicity is not a known risk factor for acute liver graft rejection and the transplant population in all studies was predominantly white. Therefore, it is not very likely that differences in ethnical background biased the results of this meta-analysis.

Although the seven studies were comparable with regard to definition of end-point, type of immunosuppression and the composition of the study population, we observed significant heterogeneity of odds-ratios between studies investigating the TNF-α –308 polymorphism. Bathgate *et al.* (3), Fernandes *et al.* (6) and Mas *et al.* (9) identified the TNFA –308 A allele as a significant risk factor for acute liver graft rejection, however this could not be confirmed by four other studies (4, 5, 7, 8). The cohort of liver transplant recipients studied by Bathgate *et al.* was enriched for autoimmune liver diseases (autoimmune hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis), accounting for the underlying etiologic cause in 85 of 144 (59%) patients (3). Moreover, Bathgate *et al.* did not describe the distribution of TNF-α genotypes among the different indications for liver transplantation despite numerous reports showing a higher prevalence of the TNFA-308 A allele in patients with autoimmune hepatitis, PBC and PSC (13, 14). With regard to the cohort of liver transplant recipients studied by Fernandes *et al.*, 9 of the 13 recipients with acute liver graft rejection were HCV-infected, and of these HCV-infected recipients, 7 patients carried the TNFA-308 A allele. The cohort of liver transplant recipients studied by Mas *et al.* was also enriched with HCV positive patients. Yee *et al.* showed that cirrhotic patients infected with HCV displayed a higher prevalence of the TNFA-308 A allele (15). Therefore, underlying etiology of end-stage liver disease may have been a significant confounder in the TNF-α gene association studies of Bathgate *et al.*, Fernandes *et al.* and Mas *et al.* Moreover, a large analysis of 210 liver transplant recipients performed by Jazrawi *et al.*, in which cases and controls were matched for underlying etiology of liver disease, revealed that the TNFA-308.A allele was not independently associated with the risk of rejection after liver transplantation.
Although, the IL-10 –1082 allele was only found to be associated with the occurrence of acute liver graft rejection after univariate analysis by Warlé *et al.*, the overall analysis combining the data of five studies revealed that the IL-10 –1082 A allele is inversely related to acute liver graft rejection (OR 0.61; 0.39-0.94 95%CI). This suggests that the statistical power of the individual gene association studies was too small to detect a moderate effect of the IL-10 –1082.A allele on the immune-response after liver transplantation. The relationship between the IL-10 –1082.A allele and low *in vitro* production of IL-10 was first described by Turner *et al.* (16). This finding was confirmed by Hoffmann *et al.* (17), whereas others found no association (18) or an opposite effect (19). Since the effect of the IL-10 –1082 promoter polymorphism on *in vitro* and thus *in vivo* cytokine production is still inconclusive, its biological effect on acute liver graft rejection remains speculative. Recently, T regulatory (Tr) cells have been found to induce IL-10-producing Th suppressor cells (Tr type 1 cells) in a cell contact-dependent manner (20). IL-10 production by these immuno-regulatory cells might be influenced by the SNP in the IL-10 gene at position –1082, affecting the allogeneic immune response after liver transplantation.

In conclusion, many cytokine gene association studies in the field of liver transplantation are not appropriately matched to control for possible confounders and do not have enough statistical power to detect moderate effects of polymorphisms in cytokine genes. Results from this meta-analysis indicate that the TNFA –308 and IL-6 –174 polymorphisms are not independently associated with acute liver graft rejection. Furthermore, polymorphisms in the TGF-β1 gene at position +869 and +915 are not significantly associated either, however their effect might be overwhelmed by the use of cyclosporin A. The IL-10 –1082 polymorphism is a significant risk factor for acute rejection in liver transplant patients, however this finding should be confirmed by a large (multicenter) study in which cases and controls are properly matched for known risk factors with regard to acute liver graft rejection such as: type and dosage of immunosuppressive drugs, age of the recipient, degree of donor-recipient human leukocyte antigen mismatch (21), and underlying etiology of liver disease (22)

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Part IV

Chapter 10

Summary and discussion

Summary and discussion

In the following section we discuss the clinical relevance of *in vivo* and *in vitro* cytokine patterns in the context of liver transplantation *(parts one and two)*. Subsequently, we discuss whether the paradigm of cytokine gene polymorphisms, influencing cytokine responses and the severity of the inflammatory response holds for liver transplantation in an animal model *(part three)*. Finally, we discuss the relevance of cytokine gene polymorphisms in human liver transplantation *(part four)*.

In vivo cytokine patterns in liver transplant patients *(part one)*

Although cytokines play a pivotal role in mediating allograft rejection, previous studies have shown that cytokines released into the circulation can not be used as specific markers of rejection or infection after liver transplantation (1). In chapter 3 we prospectively studied the clinical relevance of patterns of cytokines, cytokine receptors and adhesion molecules in serum and bile samples of liver transplant recipients during the early course after liver transplantation. Our results show that soluble intercellular adhesion molecule-1 (sICAM-1) in serum and interleukin (IL)-8 in bile are specifically increased at the onset of acute rejection episodes, whereas soluble tumor necrosis factor-receptor-II is also increased in patients with infectious complications and serum IL-6 only in patients with rejection during infection. We conclude that biliary cytokines do not provide rejection markers with higher specificity as compared to serum cytokines. Furthermore, we show that daily monitoring of serum sICAM-1 can identify patients at risk for acute rejection. Therefore acute liver graft rejection may be recognized earlier in those patients.

In vitro cytokine production profiles in liver transplant patients *(part two)*

Between individuals, differences exist in the capacity of peripheral blood mononuclear cells (PBMC) to produce cytokines after *in vitro* stimulation with a mitogen. In chapter 4 we show that patients with acute rejection after liver transplantation produce significantly higher levels of tumor necrosis factor (TNF)-α upon *in vitro* stimulation as compared to non-rejectors. Moreover, patients classified as *high TNF-α/low IL-13* producers most often rejected their liver allograft, whereas *low TNF-α/high IL-13* producers never rejected. These findings indicate that acute liver graft rejection is less likely to occur when the production of TNF-α during the allo-response is inhibited by high levels of IL-13. In previous reports, the inhibitory effect of IL-13 on inflammatory cytokine production *in vitro* by human PBMC has been described (2). An important question that has to be addressed is whether *in vitro* cytokine production profiles determined in patients after liver transplantation are a cause or consequence of the allogeneic inflammatory response. Episodes of acute rejection typically occur within the first postoperative month and maintenance immuno-

suppressive therapy is installed within the first three months after transplantation, both events occurred long before the time of blood-sampling (mean 3.5 years after transplantation), which favors the argument that cytokine production profiles are a predisposing factor rather than a consequence of rejection. This was also confirmed by a study of Bathgate *et al.* (3), who described higher TNF-α production levels by rejectors as compared to non-rejectors after *in vitro* stimulation of pre-transplantation obtained PBMC.

Polymorphisms in several cytokine genes such as TNF-α, IFN-γ, IL-10 and TGF-β, have been described to be associated with the level of *in vitro* cytokine production after stimulated cell culture of PBMC (4-7). Although these associations could not always be confirmed (8, 9), it has become accepted to denote polymorphisms of cytokine genes by their presumed association with high or low *in vitro* cytokine production. In chapter 5 we analyze the relationship between cytokine gene polymorphisms and *in vitro* cytokine production profiles, both in liver transplant recipients and healthy individuals. For healthy individuals, we observed an association between the TNF-d3 microsatellite and IL-10 –1082 single nucleotide polymorphism (SNP) with the *in vitro* production capacity of TNF-α and IL-10 respectively, whereas no significant relationship was found for other polymorphisms including: TNF-A –308; IFN-G +874; IL-10 –819 and –592; and IL-13 +2043 and –1055. For liver transplant recipients no significant associations were found. We also reviewed the literature and major differences between studies investigating the relationship between cytokine gene polymorphisms and *in vitro* production levels were found. The studies analyzed, used different cell types for stimulated culture including whole blood, isolated monocytes, and PBMC. Also, the *in vitro* stimulation protocol differed with regard to the *in vitro* stimulant, the concentration of a particular stimulant, and the length of the incubation period. Moreover, the study populations were either healthy individuals or very diverse patient groups. Given the methodological differences and contradictory results between many studies, we conclude that *in vitro* cytokine production profiles can not be deduced from particular cytokine gene polymorphisms. We suggest that cytokine gene polymorphisms are described by their localization within the gene, rather than by their presumed *in vitro* cytokine production profile.

Animal model: *in vitro* cytokine production profiles, gene polymorphisms and liver allograft survival *(part three)*

In vitro cytokine responses can be affected by factors such as age, underlying disease and immunosuppressive therapy (10-12). To avoid these possible confounding factors, we used an animal model to study strain-specific *in vitro* cytokine responses. In chapter 6 we assessed whether differences in cytokine responses between inbred rat strains can explain differences in liver allograft survival (13). Splenocytes isolated from naive rats of different inbred strains were stimulated and we found that strain-

specific TNF-α, IFN-γ, IL-6 and IL-10 responses do not predict rat liver allograft survival. This observation does not confirm the role of cytokine production profiles in naive animals as promoters of the immune response after liver allografting. A possible explanation for this finding is that naive cytokine responses are affected by the allogeneic inflammatory response after liver allografting. To test this hypothesis post-transplant cytokine responses were studied in high (Lewis; LEW) and low (Pivold Virol Glaxo; PVG) cytokine responder strains. When Brown Norway (BN) liver grafts were used, both recipient strains (LEW and PVG) showed spontaneous long-term survival. Although PVG showed low cytokine responses in naive animals, we found a small but significant up-regulation of the IFN-γ response from postoperative day 7 to 21. In LEW we found up-regulation of both T helper (Th) 1- (IFN-γ) and Th2-type (IL-6 and IL-10) cytokine responses. These data indicate that the endogenous capacity of mononuclear cells to produce cytokines upon *in vitro* stimulation is modulated during the immune response after liver transplantation. The consequence of this finding is that pre-transplant cytokine responses not necessarily reflect the cytokine production capacity after liver allografting.
In chapter 7 we address the question whether nucleotide differences in the *Tnf* gene influence *in vitro* TNF-α protein production. Among six inbred rat strains 44 nucleotide differences including 36 single nucleotide polymorphisms (SNPs), five simple sequence length polymorphisms, two deletions and one insertion were found. Our results show that polymorphisms in non-coding regulatory regions of the *Tnf* gene are not associated with the capacity to produce TNF-α in response to a mitogenic stimulus. The TNF-α gene is located within the major histocompatibility complex (MHC; class III region). Between the MHC identical strains August (AUG) and PVG, *in vitro* TNF-α responses differed significantly, but no differences in the *Tnf* nucleotide sequence were found. Together, these finding suggest that *in vitro* TNF-α responses by mononuclear cells isolated from rat spleens are regulated independent of genetic variation within the MHC including the *Tnf* locus.

Cytokine gene polymorphisms and acute human liver graft rejection *(part four)*

Although we could not establish a relationship between cytokine gene polymorphisms and *in vitro* cytokine production profiles (chapter 5 and 7), polymorphisms in regulatory regions of cytokine genes may have a modest influence on *in vivo* cytokine production levels at the site of inflammation. Cytokine gene polymorphisms may also be in linkage disequilibrium with other truly 'functional' polymorphisms or with loci or haplotypes influencing the inflammatory response. Therefore, polymorphisms in cytokine genes may act as markers of allograft rejection in liver transplant patients. In chapter 8 we analyzed whether gene polymorphisms in Th1- (IL-2 +166, IL-2 –330, IL-15 +13689, IL-15 –80, TNF-A –308, TNF-d3, IFN-G +874), Th2- (IL-4

+33, IL-4 –590, IL-6 –174, IL-10 –592, IL-10 –819, IL-10 –1082, IL-13 +2043, IL-13 –1055), and regulatory-type (TGF-B1 +869, TGF-B1 +915) cytokines are associated with biopsy-proven, human liver allograft rejection. After multivariate analysis the TGF-B1 +869 polymorphism was found to be independently associated with the occurrence of acute rejection.

Our study is one of the seven reports investigating the association between polymorphisms in cytokine genes and acute rejection in the field of liver transplantation (14-19). In chapter 9 we present an overview of this literature. Four polymorphisms in TNF-α, IL-10 and TGF-β1 cytokines genes were found to be associated with acute liver graft rejection. However these associations could not always be confirmed by other studies. A possible explanation for discrepancies between individual studies may be the lack of statistical power to detect small or moderate gene effects due to their relatively small sample sizes. To overcome this problem, we performed a quantitative meta-analysis. In the overall analysis, no associations were found between rejection and SNPs in the TNF-α (position –308), IL-6 (position –174), and TGF-β1 (positions +869 and +915) genes. In this meta-analysis the IL-10 polymorphism at position –1082 was identified as a genetic risk factor for acute human liver graft rejection.

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Appendices

Samenvatting

In de hiervolgende samenvatting wordt de klinische relevantie van *in vivo* en *in vitro* cytokine patronen met betrekking tot levertransplantatie bediscussieerd *(deel één en twee van dit proefschrift)*. Vervolgens wordt ingegaan op de vraag of cytokine genpolymorfismen van invloed zijn op de mate van cytokine productie en zodoende op de allogene ontstekingsreactie na levertransplantatie in een proefdiermodel *(deel drie)*. Tot slot wordt ingegaan op de relevantie van cytokine genpolymorfismen in het kader van humane levertransplantatie *(deel vier)*.

In vivo cytokine patronen in levertransplantatie patiënten *(deel één)*

Hoewel cytokinen een voorname rol spelen in het ontstaan van transplantaat afstoting, hebben eerdere studies laten zien dat cytokinen aanwezig in de bloedsomloop niet geschikt zijn als specifieke markers van afstoting of infectie na levertransplantatie. In hoofdstuk 3 hebben we de klinische relevantie van patronen van cytokinen, cytokinereceptoren en adhesiemoleculen onderzocht in serum- en galmonsters van levertransplantatie patiënten gedurende het vroege beloop na levertransplantatie. Onze resultaten lieten zien dat oplosbaar adhesie molecuul-1 (sICAM-1) in serum en interleukine (IL)-8 in gal specifiek verhoogd waren op het moment van acute afstoting, terwijl oplosbaar tumor necrose factor receptor-II eveneens verhoogd was in patiënten met infectieuze complicaties en serum IL-6 alleen in patiënten met afstoting tijdens infectie. We concluderen dat cytokinen in gal geen markers van afstoting zijn met een hogere specificiteit dan cytokinen in serum. Verder is gebleken dat door middel van dagelijkse monitoring van serum sICAM-1 patiënten kunnen worden geïdentificeerd die een verhoogd risico hebben om af te stoten. In deze patiënten kan acute levertransplantaat afstoting in een eerder stadium worden herkend.

In vitro cytokine productie profielen in levertransplantatie patiënten *(deel twee)*

Tussen individuen bestaan verschillen in de capaciteit van perifeer bloed mononucleaire cellen (PBMC) tot het produceren van cytokinen na *in vitro* stimulatie met een mitogeen. In hoofdstuk 4 lieten we zien dat patiënten met acute afstoting na levertransplantatie significant hogere concentraties tumor necrose factor (TNF)-α produceerden na *in vitro* stimulatie in vergelijking met niet-afstoters. Verder bleken patiënten die konden worden geclassificeerd als *hoog TNF-α/laag IL-13* producenten het vaakst hun transplantaat af te stoten, terwijl *laag TNF-α/hoog IL-13* producenten geen afstoting lieten zien. Deze bevindingen wijzen erop dat acute levertransplantaat afstoting minder snel optreedt wanneer de productie van TNF-α gedurende de allo-respons wordt geremd door hoge concentraties IL-13. In eerder onderzoek is het inhiberende

effect van IL-13 op de productie van pro-inflammatoire cytokinen door humane PBMC beschreven.
Een belangrijke vraag die dient te worden beantwoord is of *in vitro* cytokine productie profielen bepaald in patiënten ná levertransplantatie oorzaak of gevolg zijn van de allogene ontstekingsreactie. De levertransplantatie patiënten die werden onderzocht in hoofdstuk 4 maakten episodes van acute afstoting door gedurende die eerste postoperatieve maand en de immuunsuppressieve onderhoudstherapie werd ingesteld gedurende de eerste drie maanden na transplanatatie, dit alles speelde zich af ruim voordat bloedmonsters werden afgenomen voor de isolatie van PBMC (gemiddeld 3.5 jaar na transplantatie). Dit pleit voor de veronderstelling dat cytokine productie profielen een predisponerende factor zijn voor het optreden van afstoting en niet het gevolg zijn van de allogene ontstekingsreactie na transplantatie. Deze veronderstelling werd ook bevestigd door het onderzoek van Bathgate *et al.*, die hogere TNF-α concentraties beschreef bij patiënten mét afstoting na *in vitro* stimulatie van PBMC die voorafgaand aan transplantatie werden verkregen.
Beschreven is dat polymorfismen in verschillende cytokine genen zoals TNF-α, IFN-γ, IL-10 en TGF-β1 geassocieerd zijn met de mate van *in vitro* cytokine productie na celkweek met een mitogene stimulus. Hoewel deze associaties niet altijd konden worden bevestigd, is algemeen gebruik geworden om polymorfismen in cytokine genen aan te duiden volgens hun veronderstelde associatie met hoge of lage *in vitro* cytokine productie. In hoofdstuk 5 hebben we de relatie tussen cytokine genpolymorfismen en *in vitro* cytokine productie profielen onderzocht, zowel in levertransplantatie patiënten als in gezonde individuen. Bij gezonde individuen vonden we een associatie tussen de TNF-d3 microsatelliet en het IL-10 –1082 'single nucleotide polymorphism' (SNP) met de *in vitro* productie capaciteit van respectievelijk TNF-α en IL-10, terwijl geen significante relatie werd gevonden voor andere polymorfismen waaronder: TNF-A –308; IFN-G +874; IL-10 –819 and –592; and IL-13 +2043 and –1055. Bij levertransplantatie patiënten werden geen significante associaties gevonden. Daarnaast hebben we een literatuurstudie verricht waarin grote verschillen werden gevonden tussen studies die de relatie tussen cytokine genpolymorfismen en *in vitro* productie capaciteit hebben onderzocht. De bestudeerde studies maakten gebruik van verschillende celtypen voor gestimuleerde celkweek waaronder volbloed, geisoleerde monocyten en PBMC. Eveneens verschilden de *in vitro* stimulatie protocollen met betrekking tot de *in vitro* stimulus, de concentratie van de desbetreffende stimulus en de duur van de incubatie periode. Bovendien bestonden de studiepopulaties uit gezonde individuen of zeer gevarieerde patiënten groepen. Gezien de methodologische verschillen en de tegenstrijdige resultaten van de vele studies, concluderen we dat *in vitro* cytokine productie profielen niet kunnen worden afgeleid van bepaalde cytokine genpolymorfismen. We suggereren dat

cytokine genpolymorfismen dienen te worden beschreven naar hun lokalisatie in het gen en niet op basis van het veronderstelde cytokine productie profiel.

Proefdiermodel: *in vitro* cytokine productie profielen, genpolymorfismen en levertransplantaat overleving *(deel drie)*

In vitro cytokine profielen kunnen worden beïnvloed door factoren zoals leeftijd, onderliggende ziekte en immuunsuppressieve therapie. Om deze potentiële confounders te vermijden, hebben we een proefdiermodel opgezet om stam-specifieke *in vitro* cytokine responsen te bestuderen. In hoofdstuk 6 werd nagegaan of variabiliteit in cytokine responsen tussen rat inteeltstammen verschillen in levertransplantaat overleving kunnen verklaren. Splenocyten geïsoleerd uit naïeve ratten van verschillende inteeltstammen werden gestimuleerd en verschillen in stam-specifieke TNF-α, IFN-γ, IL-6 en IL-10 responsen bleken geen voorspellende waarde te hebben wat betreft levertransplantaat overleving in de rat. Deze bevinding is niet in overeenstemming met de veronderstelde rol van cytokine productie profielen bij het initiëren van de immuunrespons na levertransplantatie. Een mogelijke verklaring hiervoor is dat naïeve cytokine responsen worden beïnvloed door de allogene ontstekingsreactie na levertransplantatie. Om deze hypothese te testen werden post-transplantatie cytokine responsen bestudeerd in 'high' (Lewis; LEW) en 'low' (Pivold Virol Glaxo; PVG) cytokine responder stammen. Wanneer Brown Norway (BN) donor levers worden gebruikt, overleven beide ontvanger stammen (LEW en PVG) spontaan op lange termijn. Hoewel naïeve PVG ratten lage cytokine responsen laten zien, vonden we een kleine maar significante toename van de *in vitro* IFN-γ respons van dag 7 naar 21 na transplantatie. In LEW vonden we een toename van zowel T helper (Th) 1- (IFN-γ) en Th2-type (IL-6 en IL-10) cytokine responsen. Deze data wijzen erop dat de endogene capaciteit van mononucleaire cellen tot het produceren van cytokinen na *in vitro* stimulatie wordt gemoduleerd gedurende de immuunrespons na levertransplantatie. De consequentie van deze bevinding is dat pre-transplantatie cytokine responsen niet noodzakelijkerwijs de cytokine productie capaciteit na levertransplantatie weerspiegelen.

In hoofdstuk 7 hebben we de vraag onderzocht of nucleotide verschillen in het *Tnf* gen de *in vitro* TNF-α productie beïnvloeden. Onder zes rat inteeltstammen werden 44 nucleotide verschillen gedetecteerd waaronder 36 SNPs, vijf 'simple sequence length polymorphisms', twee deleties en één insertie. Onze resultaten lieten zien dat polymorfismen in niet-coderende regulatoire delen van het *Tnf* gen niet geassocieerd zijn met de productie capaciteit van TNF-α in respons op een mitogene stimulus. Het TNF-α gen is gelokaliseerd in het 'major histocompatibility complex' (MHC; class III region). De MHC identieke stammen, August (AUG) en PVG, verschillen significant in de mate van *in vitro* TNF-α productie, hoewel tussen deze stammen geen verschillen in de *Tnf* nucleotide sequentie werden gevonden. Concluderend,

deze bevindingen suggereren dat *in vitro* TNF-α productie door rat splenocyten onafhankelijk van genetische variatie in het MHC, waaronder het *Tnf* locus, wordt gereguleerd.

Cytokine genpolymorfismen en acute humane levertransplantaat afstoting (deel vier)

Hoewel we geen relatie hebben aangetoond tussen cytokine genpolymorfismen en *in vitro* cytokine productie profielen (hoofdstuk 5 en 7), zouden polymorfismen in regulatoire delen van cytokine genen toch van invloed kunnen zijn op *in vivo* cytokine productie ter plaatse van de ontsteking. Cytokine genpolymorfismen zouden ook in 'linkage desequilibrium' kunnen zijn met andere werkelijk 'functionele' polymorfismen of met loci of haplotypes die de ontstekingsreactie beïnvloeden. Daarom kunnen polymorfismen in cytokine genen dienen als markers van transplantaat afstoting in levertransplantatie patiënten. In hoofdstuk 8 hebben we onderzocht of gen-polymorfismen in Th1- (IL-2 +166, IL-2 –330, IL-15 +13689, IL-15 –80, TNF-A –308, TNF-d3, IFN-G +874), Th2- (IL-4 +33, IL-4 –590, IL-6 –174, IL-10 –592, IL-10 –819, IL-10 –1082, IL-13 +2043, IL-13 –1055) en regulatoir-type (TGF-B1 +869, TGF-B1 +915) cytokine genen geassocieerd zijn met histologisch bewezen, humane levertransplantaat afstoting. Na multivariate analyse was het TGF-B1 +869 polymorfisme onafhankelijk geassocieerd met het optreden van acute lever-transplantaat afstoting.

Op het terrein van levertransplantatie is ons onderzoek een van de zeven studies waarin de relatie tussen polymorfismen in cytokine genen en acute afstoting is onderzocht. In hoofdstuk 9 hebben we een overzicht laten zien van deze literatuur. Vier polymorfismen in TNF-α, IL-10 en TGF-β1 genen bleken te geassocieerd te zijn met acute levertransplantaat afstoting. Hoewel deze associaties niet altijd konden worden bevestigd door andere studies. Een mogelijke verklaring voor de discrepanties tussen de afzonderlijke studies, is het geringe statisch onderscheidings-vermogen om kleine tot middelmatige effecten van cytokine genen te detecteren ten gevolge van de relatief kleine studiepopulaties. Om dit probleem op te lossen hebben we een kwantitatieve meta-analyse uitgevoerd. In de totale analyse werden geen significante associaties gevonden tussen afstoting en SNPs in het TNF-α (positie –308), IL-6 (positie –174) en TGF-β1 (posities +869 en +915) gen. In deze meta-analyse werd het IL-10 polymorfisme op positie –1082 geïdentificeerd als een genetische risicofactor voor acute humane levertransplantaat afstoting.

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Curriculum Vitae Auctoris

The author was born in Neede, the Netherlands on 24 March 1975. After secondary school (Athenaeum), he continued his studies at the Faculty of Medicine of the Erasmus University Rotterdam. During medical school his interest in surgery was aroused when he started participating in clinical research as a research student in the Rotterdam liver transplantation team. Before starting his internship, he was offered a two-year position as PhD student at the Department of Surgery of the University Hospital Rotterdam under supervision of prof.dr. H.W. Tilanus. His PhD training focused on the role of cytokine gene polymorphisms in liver transplantation. The gene association studies were performed in collaboration with the Immunology Research Group of the University of Manchester, United Kingdom (prof.dr. I.V. Hutchinson). For his experimental studies, he was trained in the technique of liver transplantation in an animal model at the Department of Medicine of the Monash University in Melbourne, Australia (dr. B.O. Howden). In 2002 he obtained his medical degree and from October 2002 to December 2003 he worked as a resident at the Department of Surgery and the Surgical Intensive Care Unit of the Erasmus Medical Center Rotterdam. In January 2004 he started his residency in General Surgery at the Sint Francisus Gasthuis Rotterdam (dr. C.H.A. Wittens and dr. A.J.H. Kerver), to be continued at the Department of Surgery of the Erasmus Medical Center Rotterdam (prof.dr. H.J. Bonjer and prof.dr. J.N.M. IJzermans).

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