DSA are associated with more graft injury, more fibrosis and upregulation of rejection associated transcripts in subclinical rejection.

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Abbreviations:

ALT: alanine transaminase; AP: alkaline phosphatase; AST: aspartate transaminase; AMR= antibody-mediated rejection, CP: central perivenulitis; cTCMR: clinical T cell-mediated rejection; DSA: donor-specific *anti*-human leukocyte antigens antibodies; FDR: false discovery rate; gGT: *gamma*-glutamyltransferase; HAI: hepatitis activity index; HLA: *anti*-human leukocyte antigens; LAF: liver allograft fibrosis; Ltx: liver transplantation; MHC: major histocompatibility complex; NAS: non-alcoholic fatty liver disease activity score; NHR: no histological rejection; PCA: principal component analysis; RAI: rejection activity index; RT-PCR: real-time polymerase chain reaction; subTCMR: subclinical T cell-mediated rejection; TCMR: T cell-mediated rejection; ULN: upper limit of normal

Abstract

Background: Subclinical T cell-mediated rejection (subTCMR) is commonly found after liver transplantation and has a good short-term prognosis, even when it is left untreated. Donor-specific antibodies (DSA) are putatively associated with a worse prognosis for recipient and graft after liver transplantation.

Methods: To assess the immune regulation in subTCMR grafts, gene expression of 93 transcripts for graft injury, tolerance and immune regulation was analyzed in 77 biopsies with “no histological rejection” (NHR; n=25), “clinical TCMR” (cTMCR; n=16) and subTCMR (n=36). In addition, all available subTCMR biopsies (n=71) were tested for DSA with bead assays.

Results: SubTCMR showed heterogeneous and intermediate expression profiles of transcripts that were upregulated in cTCMR. Graft gene expression suggested a lower activation of effector lymphocytes and a higher activation of regulatory T cells in grafts with subTCMR compared to cTCMR.

DSA positivity in subTCMR was associated with histological evidence of more severe graft inflammation and fibrosis. This more severe DSA+ associated graft injury in subTCMR was converged with an upregulation of cTCMR associated transcripts. In non-supervised analysis DSA positive subTCMR mostly clustered together with cTCMR, while DSA negative subTCMR clustered together with NHR.

Conclusion: T cell-mediated rejection seem to form a continuum of alloimmune activation. Although subTCMR exhibited less expression of TCMR associated transcript, DSA positivity in subTCMR was associated with an upregulation of rejection associated transcripts. The identification of DSA positive subclinical

rejection might help to define patients with more inflammation in the graft and development of fibrosis.

Key words: T cell-mediated rejection, liver transplantation, subclinical rejection, immunosuppression, donor specific antibodies

Introduction

The increase in patient and graft survival after liver transplantation (Ltx) has mostly been achieved by improvements in the early post-transplantation phase, while the decline due to chronic graft failure showed no further improvement over the last decades[1]. Side effects of chronic immunosuppression, disease recurrence and insufficient control of chronic alloreactivity are considered to be main causes of the stagnating long-term prognosis[1,2]. The last two issues mostly contribute to subclinical histological changes in the majority of liver biopsies late after transplantation[3]. The relevance of these subclinical changes, which are only traceable in protocol liver biopsies, mostly remains unclear.

Subclinical T cell-mediated rejection (subTCMR) describes the presence of the histological features of T cell-mediated rejection (TCMR) but without relevant elevations of liver enzymes. Although arbitrarily chosen, the two times upper limit of normal (ULN) as commonly used threshold for liver enzymes was associated with the cumulative patient survival after early rejection[4]. SubTCMR is commonly found after Ltx in up to 60% early (≤4 weeks) and in approximately 25% of protocol biopsies later (≥3 months) after transplantation, while clinical TCMR (cTCMR) is more prevalent earlier after transplantation. SubTCMR can be found in protocol biopsies with or without previous cTCMR and in longitudinal protocol biopsies[5]. So far subTCMR has a good short to medium term prognosis even if left untreated[5-8]. However, long-term consequences are largely unknown.

The immunological mechanisms protecting liver grafts in subTCMR are largely unknown. The histological immunophenotyping suggested a stricter regulation of cytotoxic T cells in grafts with subTCMR compared to cTCMR[5,9].

SubTCMR after kidney transplantation has a good prognosis[10], although rejection and graft injury associated transcripts are upregulated compared to grafts without rejection but less extensive than in clinical rejection[11,12]. The subsequent appearance of donor-specific *anti*-human leukocyte antigens antibodies (DSA) with a transplant glomerulopathy after subTCMR showed a higher risk for graft loss after kidney transplantation[10].DSA after Ltx are putative risk factors for a reduced graft and patient survival. Furthermore, DSA are associated with graft fibrosis and chronic liver allograft rejection[13-21].

Our hypothesis was to find a similar gradual upregulation of rejection and injury associated transcripts in liver grafts with subTCMR as it has been described for renal grafts[11,12]. Furthermore, intrahepatic transcriptional levels should be elucidated with regard to the presence of DSA.

Material and Methods

Subjects

We included all liver recipients without a replicative viral hepatitis (HCV-RNA or HBs-Ag negativity) who underwent at least one liver biopsy and agreed to participate in our prospective liver allograft biorepository since 2008. Liver biopsies came from our protocol biopsies program (intended time points: months 3+6+12 and then annually), or from patients with a liver biopsy because of elevated liver enzymes. Participation in the protocol biopsy program was voluntary and offered to all liver transplanted patients without contraindications, e.g. dilatated bile duct, thrombocytopenia etc., even when they were transplanted before 2008. Likewise, all patients with a liver biopsy because of elevated liver enzymes were asked for a participation in the biorepository. Approximately ¾ of biopsies in the repository are protocol biopsies and ¼ biopsies for cause. Up to 30% of patients participated in the protocol biopsy program.Only patients with biopsy proven subTCMR, cTCMR and comparators without rejection and with normal graft function (“no histologic rejection” (NHR)) were selected for this study.

The study was approved by the local Ethics Committee (protocol number 933 for project Z2 of comprehensive research center 738). Written informed consent was obtained from all patients.

Histological grading and staging

Sections of 2 µm thickness were stained with haematoxylin and eosin, elastic van gieson stain, periodic acid–Schiff stain, silver stain, Berlin blue stain and rhodanine stain. Histological examination and scoring for the rejection activity index (RAI),

inflammation grade and fibrosis stage, central perivenulitis (CP), portal microvasculitis, ductular reaction[22], fatty liver disease[23] and liver allograft fibrosis (LAF) score[24] was performed by experienced liver pathologists in a blinded fashion as described previously.

Detection of donor-specific *anti*-human leukocyte antigens antibodies.

Plasma samples for DSA detection were taken within 24 hours around the liver biopsy and cryopreserved at -80° C. The recipient plasma were screened for the presence of HLA class I/II antibodies using mixed HLA antigen-charged polysterene beads (LIFECODES LifeScreen Deluxe-LMX test Gen-Probe-Immucor, Stanford, CT, USA) and a multichannel flow array (Luminex, Austin, TX) as described previously[25]. A specification of HLA antibodies in sera with a positive screening result was performed using class I/class II single-antigen beads (LIFECODES Single Antigen-LSA test Gen-Probe-Immucor, Stanford, CT, USA). The tests were performed according to the manufacturer's instructions. The incidence of DSA positivity was analyzed using MFI threshold of 1000 or more for the plasma antibodies against HLA and a positive match with donor HLA typing.

Definition of subclinical and clinical rejection

SubTCMR and cTCMR were defined as recently published[5]. In short, subTCMR was defined by a Banff RAI≥1+1+1 (portal, bile duct, venous endothelial inflammation) in the absence of relevant liver enzyme elevations. We included only those biopsies that fulfilled all three criteria to exclude borderline TCMR. CTCMR was defined as

RAI≥1+1+1 with relevant liver enzyme elevations (Fig. S1). The cut-off for a relevant liver enzyme elevation was defined as an elevation above twice ULN (≥2xULN) of at least one of the following liver enzymes: alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (AP)[9]. For the definition of subTCMR levels of the gamma-glutamyltransferase (gGT) had to be stable or declining, even if elevated above twice ULN, in the weeks and months before the protocol biopsy, while an increasing gGT before the biopsy showing TCMR was considered as cTCMR. This selection was chosen to distinguish better between persistent structural biliary complications and TCMR.

Biopsies of patients with evidence of disease recurrence, viral replication or bacterial infection were excluded.

The control group of no histologic rejection (NHR) consisted of protocol biopsies without TCMR (RAI≤1), without relevant inflammation, no relevant fibrosis (Ishak F≤1, each LAF score components≤1) and liver enzymes within the normal range (Fig. S1).

Statistics

Statistical analysis was performed with SPSS version 15.0 and GraphPad Prism 5.01 software. The Mann-Whitney U test was used to compare quantitative data between two independent groups and the Kruskal-Wallis test with Dunn's multiple comparison post hoc test for more than two groups. The Wilcoxon test was used to compare paired groups. The Fisher's exact test was used to compare contingency tables with two groups and the Chi square test was used to compare more than two groups.

Principal component analysis (PCA) and heat maps of -ΔCt values were performed using Qlucore Omics Explorer v3.3 (Qlucore, Lund, Sweden). For analysis p values

were set of ≤0.049 to compare groups to each other for two group comparisons (t-test) and multi group comparison (F-test) (ANOVA). False discovery rate (FDR) calculated for correction of multiple t testing <0.1 was considered significant for all Qlucore analysis.

For pathway analysis with Ingenuity Pathway Analysis (IPA, QIAGEN) the fold changes of the median expression value were used to compare groups NHR vs cTCMR/subTCMR, subTCMR vs cTCMR and DSA+ subTCMR vs DSA- subTCMR.

P-values below 0.05 (two-tailed) were considered statistically significant in all analyses.

Further material and methods are listed in the supplemental information.

Results

Patients and biopsies.

This study is based on our prospective liver biopsy biorepository after liver transplantation. Out of the 232 recruited patients, in whom 737 liver biopsies were taken, liver biopsies fulfilling the criteria of subTCMR were selected as entity of interest and with cTCMR and NHR as comparators (Figure 1). Clinical characteristics of subTCMR and cTCMR of our cohort were published recently[5]. With the available short-medium term follow-up NHR, subTCMR and cTCMR exhibited no significant progression of fibrosis (Fig. S2). However, these results have to be interpreted cautiously, because patients could have NHR, subTCMR or cTCMR in subsequent biopsies.

Only biopsies with mRNA isolated from cryo-conserved biopsies, available in 100 of 179 biopsies, were used for this graft gene expression study. To reduce potential confounders, samples with available mRNA were matched in all groups, as far as possible without reducing samples numbers too much, for age and time after transplantation. SubTCMR and cTCMR were further matched for RAI and fibrosis (Fig. 1, Table 1, Table S1, Fig S3).

Patients of this gene expression study were transplanted 1988-2015 and liver biopsies were taken 2009-2016. NHR had no relevant elevation of liver enzymes and only minimal histological abnormalities. SubTCMR was characterized by normal or only marginally elevated liver enzymes with mild-moderate histological abnormalities. CTCMR exhibited the highest liver enzyme elevation with the most prominent histological abnormalities (Table 1). Differences in immunosuppressive regimen between the groups were most likely related to differences in time after transplantation as well as the era during which the patient was transplanted.

Intermediate expression of genes associated with rejection, immunoregulation and endothelial cells in grafts with subTCMR.

Of all graft RNA samples 77 (NHR=25, subTCMR=36, cTCMR=16) were selected for gene expression analysis.

At first the expression of 93 transcripts for rejection, endothelial cells, immunoregulation including T cell exhaustion and operational tolerance after Ltx (Table S2)[26-29] was determined in these 77 liver allograft biopsies irrespective of the DSA status.

The expression of more than half of the 93 transcripts (54/93; 58%) was significantly different in the three groups NHR, subTCMR and cTCMR studied according to the principal component analysis (PCA) ($p<0.05$; FDR<0.079; Figure 2A+B; Table S3). Thereby, two clusters, one with transcripts being upregulated (red cluster) and one being downregulated (green cluster) in cTCMR, could be identified (Fig. 2B). Marked differences in the graft gene expression were noted between cTCMR and NHR. In contrast, gene expression in subTCMR broadly overlapped with both other states and was inhomogeneous within the subTCMR group itself mostly in the red cluster (Fig. 2A+B). In contract subTCMR and NHR exhibited similar expression of transcripts from the green cluster, which were downregulated in cTCMR.

In the pairwise molecular pathways analysis of NHR, subTCMR and cTCMR the same pathways were overexpressed in all three comparisons (Table 2). The results supported the notion that subTCMR was characterized rather by a less extensive expression of the same set of transcripts that were upregulated in cTCMR, than by the expression of unique transcript set.

We recently quantified the infiltration of T cells and Treg in subTCMR, cTCMR and NHR in histology[5]. The current gene expression analysis confirmed the histological

finding of no significant overall differences in the intrahepatic infiltration of regulatory (FOXP3) and total T cells (CD3) between subTCMR and cTCMR, all of which from the red cluster (Fig. 2C). However, the intrahepatic expression of granzyme B (red cluster), an effector protease e.g. of cytotoxic T (CD8) and NK cells, was significantly lower in subTCMR and NHR compared to cTCMR, although CD3e and CD8A expression (red cluster) is not significantly different in subTCMR and cTCMR. Furthermore, the expression of S1PR1, the sphingosine-1-phosphate receptor 1 (S1PR1, green cluster), which for instance is downregulated after T cell activation and then leads to a retention of activated T cells in inflamed tissue and which is also expressed by endothelial cells[30], was significantly higher in subTCMR compared to cTCMR. In contrast, LRRC32 (green cluster), a specific Treg activation marker[31], is significantly higher in subTCMR and NHR compared to cTCMR, although the expression of the Treg lineage marker FOXP3 is not significantly different in subTCMR and cTCMR (Fig. 2C). In additional to LRRC32 and S1PR1, RORC is the only other transcript from the green cluster that is downregulated in cTCMR.

Next subgroups in subTCMR were be identified, because the expression of the selected transcript sets is not homogenous in subTCMR (Fig. 2B). First, subTCMR was dissected into those with normal AST, ALT and AP (24/36; 67%) and those with marginal elevations of these liver enzymes (>1 and ≤2x ULN; 12/36; 33%). However, expression of the 93 transcripts was not significantly different between these two subgroups in the PCA. Next the liver allograft gene expression were analyzed regarding the DSA status of the recipient.

More severe graft hepatitis and liver fibrosis in DSA positive subTCMR.

All blood samples paired to liver biopsies in our program were screened for DSA (365 samples of 185 patients). Of the 80 subTCMR biopsies in our program DSA could be

assessed in 71 but not in the remaining 9 samples, because of incomplete HLA typing of the donor. DSA were found in 19/71 (27%; DSA+) and not present in 52/71 (73%; DSA-) subTCMR samples (Table 3, Fig. 3A). DSA frequency in subTCMR was comparable to the centers background, lower than in cTCMR and higher than in NHR (Fig. 3A). DSA specificities were mostly targeted against HLA class II DQ epitopes with no significant enrichment of certain DSA specificities in subTCMR compared to the whole biopsy program (Chi square test including specificities against A; A+C; DQ; DR; DQ+DR; others: p=0.460) (Fig. 3B).

Regarding the time course of DSA presence 11/19 (58%) subTCMR samples contained persisting DSA, which were detectable in all sequential samples starting approximately 2-3 months after transplantation. Unfortunately, we had no systematic and complete information about the DSA status before or at transplantation. *De novo* DSA (in comparison to the initial post-transplant samples) were found in 5/19 (26%) samples. The remaining 3/19 (16%) DSA+ subTCMR samples were the first available samples after transplantation (data not shown). So, the time course could not be determined in detail, although DSA frequency was increasing with time after transplantation in our center (data not shown).

The demographic and clinical parameters of DSA+ and DSA- subTCMR patients were not significantly different with a trend to a more frequent use of cyclosporine A in DSA+ subTCMR (Table 3, Fig. S4). However, the presence of DSA was associated with slightly higher histopathological scores for portal and lobular inflammation, RAI and fibrosis in subTCMR (Fig. 3C, Table 3). Additionally, DSA+ subTCMR also exhibited marginally higher scores for ductular reaction and nodular regenerative hyperplasia (Table 3). Unfortunately, longitudinal follow-up biopsies of DSA+ subTCMR were only available in a limited number of patients thereby preventing a reliable estimation of the long-term prognosis.

Up-regulation of genes mostly associated with rejection and T cell exhaustion in DSA positive subTCMR.

Next, graft gene expression in subTCMR was compared regarding the DSA status, which could be determined in paired blood samples of 28/36 (78%) subTCMR biopsies with available gene expression data, while paired blood samples or a complete donor HLA typing were unavailable in the remaining 8/36 (22%) samples. DSA were then detectable in 8/28 (29%) subTCMR samples. Histological characteristics of this subTCMR sub-cohort with available graft RNA is summarized in Table S1 and showed that selected samples are representative for the total subTCMR cohort (Table 3).

Of the 93 selected transcripts 34 (37%) were significantly up-regulated in DSA+ compared to DSA- subTCMR ($p<0.033$; FDR<0.1; Fig. 4A+B; Table S4), while no transcript was down-regulated in DSA+ subTCMR. Thereby, differentially expressed transcripts were mostly associated with rejection (19/39 (49%) transcripts were differentially regulated) and T cell exhaustion (6/15 (40%) transcripts), less immunoregulation (6/18 (33%) transcripts) and endothelial cell markers (3/16 (19%) transcripts) and none of the operational tolerance markers were up-regulated in DSA+ subTCMR.

In the molecular pathway analyses the same pathways were overexpressed in DSA+ subTCMR as in cTCMR (Table 2). Furthermore, DSA+ subTCMR clustered mostly with cTCMR (violet cluster) and DSA- subTCMR with NHR (turquoise cluster) in a non-supervised analysis ($p <0.05$; FDR<0.05; Fig. 4C; Table S5).

Discussion

The understanding of rejection processes after Ltx beyond acute TCMR and spontaneous operational tolerance is far more limited than after kidney transplantation. So, this is, to our knowledge, the first graft gene expression analysis in subTCMR after Ltx.

SubTCMR seems to have a good prognosis even when left untreated after liver and kidney transplantation[5,7,10]. After kidney transplantation the subsequent appearance of DSA in the presence of a transplant glomerulopathy worsens the prognosis of subTCMR[10]. DSAs are closely associated with renal graft damage. This association seems to be weaker in liver grafts[32]. However, while DSA were associated with a progressive liver graft fibrosis and graft loss[13-21], DSA are also found in patients with spontaneous operational tolerance after Ltx and with stable graft function without any immunosuppression[33-35].

DSA after kidney transplant can be further characterized by C1q binding in blood tests and C4d deposition in histology both leading to a worse prognosis[36]. This association is much weaker after Ltx. The most promising candidate is IgG3 DSA, while the detection of C4d deposition remains problematic in paraffin embedded tissue[14]. The majority of DSA+ liver biopsies (>98%) in our center were C4d negative and the positive biopsies had a C4d deposition that was not suggestive for antibody mediated rejection (AMR). One third of DSA+ subTCMR biopsies (n=7) also exhibited histological features of “possible” chronic AMR. Gene expression was available in only three of these subTCMR/AMR biopsies preventing further analysis.

Although our cohort is too small and the current follow-up too short for an estimation of the prognosis of DSA+ subTCMR after Ltx, it is interesting that DSA positivity is associated with mildly higher scores for graft injury. The finding of slightly more liver

fibrosis in all compartments in DSA+ subTCMR points to a progressive tissue damage in these patients that is not biased by time intervals between transplantation and biopsy (Table 3).

Of course, the current study cannot unravel whether DSA are causative for graft injury, or whether the humoral immune system is sensitized towards MHC II molecules in a bystander fashion by the upregulation of MHC II in inflamed liver grafts as summarized in the second hit hypothesis[22,32].

Of note, we carefully excluded all patients with evidence of an infectious (viral and bacterial) trigger for subTCMR, overlapping disease recurrence, e.g. of primary sclerosing cholangitis and primary biliary cholangitis, or accompanying non-alcoholic steatohepatitis. However, patients with a history of bile duct complications were not excluded as long there was no evidence of an acute obstructive cholestasis or cholangitis, because associations of humoral alloreactivity and bile duct complications are occasionally reported in some but not all studies[16,37]. The slightly higher scores for ductular reaction in DSA+ subTCMR could be a manifestation of ischemic biliary injury related to antibody-mediated injury to the peribiliary vascular plexus. The primarily arterial blood supply and the limited regenerative capacities of bile ducts predispose to biliary damages due to transplant vasculopathy[38]. Nodular regenerative hyperplasia, which appeared to be more common in the DSA+ biopsies, could also reflect antibody-mediated injury to small portal veins and/or sinusoids.

Of note, the differences of histological scores between DSA+ and DSA- subTCMR are usually mild, not more than one score point. This suggests that graft damaging processes proceed rather slowly in untreated subTCMR. However, the constellation of additional hepatitis features beside the rejection characteristics in the portal tract in the presence of DSA seem to mark patients that may deserve a closer surveillance in the future, even when liver enzymes are normal.

Graft gene expression was assessed in subTCMR to help to understand why the clinical phenotype remains subclinical, although histological criteria of TCMR are fulfilled. The biopsies that were used for gene expression analysis here started earliest at month 2 and the majority of biopsies were taken beyond the first year after transplantation. Hence, injury-repair responses to graft implantation stresses, as found in week 6 kidney biopsies, should be no bias[39]. Differences of the rejection groups in the time point after transplantation could not be compensated without dramatically reducing the sample number. NHR and cTCMR biopsies are more frequent earlier after transplantation, while subTCMR has a relatively stable incidence over many years (Table 1)[5]. We tried to minimize a bias of the histological severity of rejection between subTCMR and cTCMR by carefully matching samples in both groups in the gene expression data set (Fig. 1, Table 1). In summary, we found that rejection or inflammation associated transcripts exhibited a gradual increase from NHR, escalating through subTCMR and culminating in cTCMR. A similar gradual increase is observed in subTCMR after kidney transplantation with larger gene sets and transcriptome analyses, too[11,12]. On the basis of the inflammation associated transcripts the two rejection phenotypes seem to form a continuum of alloimmune activation in liver and kidney transplants[11]. However, there was no significant difference in the graft gene expression of those with and without marginal liver enzyme elevation within the group of subTCMR.

Interestingly, three transcripts, LRRC32, S1PR1 and RORC, did not follow this continuum. All of which were similarly expressed in NHR and subTCMR and downregulated in cTCMR. LRRC32 is selectively upregulated in Treg but not in effector T cells upon activation[31]. RORC is expressed at least in two isoforms. Unfortunately, no available primer selectively amplifies the RORγt isoform, the lineage marker for pro-inflammatory Th17 cells. So the RORC transcripts are most

likely derived from hepatocytes, where the RORγ isoform, involved in regulation of circadian rhythms, is also expressed[40]. S1PR1 is expressed by endothelial cells and lymphocytes and is involved in lymphocyte trafficking. Thereby, a S1PR1 downregulation, as seen here during cTCMR compared to subTCMR and NHR, is observed after T cell activation and leads to a retention of activated T cells in inflamed tissue[30]. Similarly, S1PR1 is downregulated in tissue resident memory lymphocytes[41]. However, we cannot attribute the differential S1PR1 expression to a single cell type in our approach. The upregulation of GZMB in cTCMR with similarly low levels in subTCMR and NHR also argues for a higher activation state of intrahepatic cytotoxic lymphocytes (T- and NK cells) in cTCMR. However, granzyme positive cells were similarly increased in cytospins from fine needle aspiration biopsies during subTCMR and cTCMR compared to NHR[42].

The intrahepatic gene expression of CD3, CD8 and FOXP3 backed the results from our previous histological immunophenotyping showing no overall difference in liver infiltration of T cells and Treg in subTCMR and cTCMR[5]. In summary, gene expression in subTCMR seems to imply a lower activation state of effector lymphocytes and a higher activation state of Tregs in the liver allograft. Such mechanisms may inhibit graft injury and keep liver enzymes mostly in the normal range. Interestingly, the same molecular pathways that characterize cTCMR are upregulated in DSA+ subTCMR as well. Hence, within the continuum of alloimmune activation DSA+ subTCMR seem to range closer to cTCMR, while DSA- subTCMR seem to range closer to NHR.

A very recent publication found high expression of gene modules enriched in rejection associated transcripts in late liver allograft biopsies with progressive fibrosis in median 13 years after transplantation[43]. These results from Londono *et al.* suggest a graft damaging potential of subclinical inflammation in the long run. According to

the subclinical expression of rejection associated transcripts we would hypothesize that subTCMR without DSA have a lower risk for long term graft damage than DSA+ subTCMR. This has to be proven in the next 5-10 years.

Although, we prospectively collected the biomaterial for this study over a period of 8-10 years, the donation of biomaterial was voluntary, which could introduce a bias by selection of the most compliant and cooperative patients. Furthermore, protocol biopsies, where the majority of subTCMR were coming from, were only performed voluntarily, again biasing towards the most compliant and most healthy patients. Patients with increased biopsy risks, e.g. low platelets and dilatated bile ducts, were not selected for protocol biopsies, because clinical benefits of protocol biopsies are not convincingly proven. Nonetheless, the comparisons within the group of subTCMR should not be affected by this selection bias, because patients fulfilled the same selection criteria.

In summary, subTCMR after Ltx is a rather inhomogeneous group, in histology and graft gene expression. Thereby, a humoral allo-sensitization as indicated by the appearance of DSA was associated with more subclinical graft injury, more graft fibrosis and upregulation of cTCMR associated transcripts. Hence, the appearance or persistence of DSA in the context of subTCMR should prompt a closer monitoring and reevaluation of the immunosuppressive regimen in these patients.

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Table 1: Demographic and clinical characteristics of patients and liver biopsies available for intrahepatic gene expression analysis.

	N	Age at biopsy (yrs)	Time since Tx (yrs)	Sex (male)	Immunosuppression prior to biopsy			Therapy	RAI	HAI	LAF	ALT (times ULN)	N	AST times ULN)	N	AP (times ULN)	N
					CNI/mTOR	MMF	Predni solon	Mono/ duo/triple									
NHR	25	50.0 (22-69)	1.00 (0.45-7.11)	48%	64% Tac, 36% CsA	88%	72%	4/32/64 %	1.0 (0-1)	1.0 (0-2)	0.0 (0-3)	0.44 (0.18-1.09)	25	0.63 (0.39-1.00)	25	0.56 (0.23-1.07)	21
subTCMR	36	52.0 (21-66)	1.96 (0.45-27.58)	67%	47% Tac, 47% CsA, 3% no CNI, 3% Everolimus	86%	83%	3/19/75 %	3.0 (3-6)	2.5 (0-7)	1.0 (0-8)	0.47 (0.13-1.80)	35	0.74 (0.29-1.54)	35	0.75 (0.23-1.30)	31
cTCMR	16	45.5 (18-76)	0.68 (0.19-12.70)	31%	44% Tac, 56% CsA	94%	94%	0/13/88 %	4.0 (3-6)	4.0 (2-9)	1.0 (0-2)	3.35 (2.10-11.80)	16	2.50 (1.50-5.50)	16	2.15 (1.20-8.60)	16

Medians with ranges or percentages are presented where appropriate. ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate transaminase; CNI, calcineurin inhibitor; CsA, Cyclosporine A; cTCMR, clinical T cell-mediated rejection; HAI, Hepatic Activity Index; LAF, Liver Allograft Fibrosis; MMF, Mycophenolate mofetil; N, number; NHR, no histologic rejection; RAI, rejection activity index; subTCMR, subclinical T cell-mediated rejection; Tac, Tacrolimus; Tx, transplantation; ULN, upper limit of normal; yrs, years

Table 2: Molecular pathways overrepresented in intra-graft expression profiles of pairwise comparisons.

Ingenuity Canonical Pathways with p values<10^{-10}	p value				Molecules
	NHR vs cTCMR	NHR vs subTCMR	subTCMR vs cTCMR	DSA+ subTCMR vs DSA- subTCMR	
Th1 and Th2 Activation Pathway	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IRF1,CD3E,IL2*,TGFB1,IFNG,HLA-DQB1,IL6,KLRC1,CD274,HLA-DRA,HAVCR2,GATA3,CD8A,CD40LG,HLA-DMA,STAT1,IL10,SOCS1,CD86,TBX21,S1PR1
Th1 Pathway	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IRF1,CD3E,IL2*,IFNG,HLA-DQB1,IL6,KLRC1,CD274,HLA-DRA,HAVCR2,GATA3,CD8A,CD40LG,HLA-DMA,STAT1,IL10,SOCS1,CD86,TBX21
T Helper Cell Differentiation	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IL2*,TGFB1,IFNG,HLA-DQB1,IL6,HLA-DRA,GATA3,CD40LG,FOXP3,HLA-DMA,STAT1,IL17A,IL10,RORC,CD86,TBX21
Type I Diabetes Mellitus Signaling	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IRF1,CD3E,IL2*,IFNG,HLA-DQB1,HLA-F,HLA-DRA,GZMB,HLA-DMA,STAT1,BCL2,SOCS1,CD86
Communication between Innate and Adaptive Immune Cells	< 0.00001	< 0.00001	< 0.00001	< 0.00001	CD83,IL2*,CD8A,CD40LG,IFNG,CXCL10,IL10,IL6,CD86,HLA-F,HLA-DRA,CXCL8
Allograft Rejection Signaling	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IL2*,CD40LG,IFNG,HLA-DQB1,HLA-DMA,IL10,CD86,HLA-F,HLA-DRA,GZMB
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IL2*,CD40LG,TGFB1,IFNG,HLA-DQB1,HLA-DMA,IL17A,IL10,IL6,CD86,HLA-DRA
Autoimmune Thyroid Disease Signaling	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IL2*,CD40LG,HLA-DQB1,HLA-DMA,IL10,CD86,HLA-F,HLA-DRA,GZMB
Graft-versus-Host Disease Signaling	< 0.00001	< 0.00001	< 0.00001	< 0.00001	IL2*,IFNG,HLA-DQB1,HLA-DMA,IL6,CD86,HLA-F,HLA-DRA,GZMB
Th2 Pathway	< 0.00001	< 0.00001	< 0.00001	< 0.00001	GATA3,CD3E,IL2*,TGFB1,IFNG,HLA-DQB1,HLA-DMA,IL10,CD86,TBX21,HLA-DRA,S1PR1
Hepatic Fibrosis / Hepatic Stellate Cell Activation	< 0.00001	< 0.00001	< 0.00001	< 0.00001	CXCL9,COL4A1,CD40LG,TGFB1,IFNG,MMP9,STAT1,BCL2,IL10,IL6,HGF,CXCL8

*not for DSA+ subTCMR vs DSA- subTCMR

Table 3: Demographic and clinical characteristics of patients and liver biopsies with subTCMR.

		subTCMR (total)		DSA+ subTCMR (A)		DSA- subTCMR (B)		p value (A vs B)
		Median (range)	N	Median (range)	N	Median (range)	N	
Patient number		60		13		44		
Age at Tx (yrs)		47 (10-68)		45 (12-62)		50 (17-68)		0.676
Sex (male)		65%		77%		66%		0.520[a]
Biopsy number		80		19		52		
Age at biopsy (yrs)		50 (21-76)		50 (22-65)		52 (21-76)		0.790
Time since Tx (yrs)		1.47 (0.25-27.58)		2.07 (0.49-18.52)		1.97 (0.25-11.25)		0.258
Immunosuppression prior to biopsy	**CNI/mTOR**	45% Tac, 53% CsA, 1% Everolimus, 1% no CNI		26% Tac, 74% CsA		54% Tac, 44% CsA, 2% no CNI		0.058[b]
	Mycophenolate	88%		95%		87%		0.673[a]
	Prednisolon	78%		68%		85%		0.178[a]
	Therapy: mono/ duo/ triple	3/ 26/ 70%		8/ 31/ 61%		2/ 25/ 73%		0.564[a]
ALT (times ULN)		0.51 (0.13-1.97)	79	0.53 (0.31-1.80)	19	0.51 (0.13-1.97)	52	0.184
AST (times ULN)		0.74 (0.29-1.90)	79	0.83 (0.51-1.90)	19	0.74 (0.29-1.80)	52	0.307
AP (times ULN)		0.75 (0.22-1.93)	73	0.92 (0.48-1.93)	18	0.71 (0.22-1.90)	47	0.076
gGT (times ULN)		0.65 (0.15-11.82)	79	0.58 (0.26-11.82)	19	0.68 (0.15-8.25)	52	0.706
Bilirubin (times ULN)		0.48 (0.14-27.24)	79	0.52 (0.29-1.33)	19	0.48 (0.14-27.24)	52	0.317
RAI	**total**	3 (3-8)	80	**4 (3-8)**	19	**3 (3-6)**	52	**0.015**
	portal	1 (1-3)	80	**2 (1-3)**	19	**1 (1-3)**	52	**0.014**
	venous endothelial	1 (1-3)	80	**1 (1-2)**	19	**1 (1-3)**	52	**0.044**
	bile duct	1 (1-3)	80	1 (1-3)	19	1 (1-2)	52	0.114
Hepatitis activity index	**total**	3 (0-10)	80	**3 (1-6)**	19	**2 (0-10)**	52	**<0.001**
	A (interface hepatitis)	0 (0-2)	80	1 (0-2)	19	0 (0-2)	52	0.151
	B (confluent necrosis)	0 (0-5)	80	0 (0-1)	19	1 (1-2)	52	0.468
	C (lobular inflammation)	1 (0-2)	80	**1 (0-2)**	19	**0 (0-2)**	52	**0.003**
	D (portal inflammation)	1 (0-3)	80	**2 (1-3)**	19	**1 (0-3)**	52	**0.001**

Central perivenulitis		0 (0-4)	80	**0 (0-2)**	19	**0 (0-4)**	52	**0.007**
Portal microvasculitis		0 (0-2)	76	**1 (0-1)**	17	**0 (0-2)**	50	**0.004**
Ductular reaction		1 (0-2)	76	**1.0 (0-2)**	17	**0.5 (0-2)**	50	**0.001**
Nodular regenerative hyperplasia		0 (0-1)	76	**0 (0-1)**	17	**0 (0-1)**	50	**<0.001**
NAS	**total**	0 (0-5)	79	**0 (0-2)**	18	**0 (0-1)**	52	**0.019**
	steatosis	0 (0-2)	79	**0 (0-1)**	18	**0 (0-1)**	52	**0.021**
	ballooning	0 (0-2)	79	0 (-)	18	0 (-)	52	1.000
	lobular inflammation	0 (0-1)	79	**0 (0-1)**	18	**0 (-)**	52	**0.003**
Ishak fibrosis stage	**F (periportal fibrosis)**	1 (0-5)	80	**1 (0-5)**	19	**1 (0-5)**	52	**0.033**
Liver allograft fibrosis score	**total**	1 (0-8)	80	**2 (0-8)**	19	**1 (0-5)**	52	**0.002**
	portal tract fibrosis	1 (0-3)	80	**1 (0-3)**	19	**1 (0-3)**	52	**0.010**
	sinusoidal fibrosis	0 (0-2)	80	**0 (0-2)**	19	**0 (0-1)**	52	**<0.001**
	perivenular fibrosis	0 (0-3)	80	**0 (0-3)**	19	**0 (0-2)**	52	**0.031**

Medians with ranges or percentages are presented where appropriate. ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate transaminase; CNI, calcineurin inhibitor; CsA, Cyclosporine A; DSA, donor-specific anti-human leukocyte antigens antibodies; gGT, gamma-glutamyltransferase; N, number; NAS, nonalcoholic fatty liver disease Activity Score; RAI, rejection activity index; subTCMR, subclinical T cell-mediated rejection; Tac, Tacrolimus; Tx, transplantation; ULN, upper limit of normal; yrs, years. a: Fisher-Exact-Text; b: Chi square test. All other comparisons between A and B are done with the Mann-Whitney U test.

Figure Legends

Figure 1: Patient selection strategy.

Flow chart outlining availability and selection of biomaterial for this study. The definition of no histological rejection (NHR), subclinical T cell-mediated rejection (subTCMR) and clinical TCMR (cTCMR) is outlined in more details in the method section. (*matching as far as possible – see Table 1).

Figure 2: Intermediate expression of genes associated with rejection, immunoregulation and endothelium in grafts with subTCMR.

Analysis of intrahepatic gene expression of 93 markers for rejection (RM), endothelial cells (ECM), immunoregulation (IM), T cell exhaustion (TCEM) and spontaneous operational tolerance (SOTM). A) Principal component analysis (PCA), calculated using –ΔCt values of all 93 measured genes (Table S2), showed a distinct clustering of biopsies with no histologic rejection (NHR; n=25) and clinical T cell-mediated rejection (cTCMR; n=16), while those with subclinical TCMR (subTCMR; n=36) overlapped with both others. B) Heat map summarizing the genes with significantly different expression upon PCA in the three rejection states ($p<0.05$; FDR<0.079). Transcripts are also listed in Table S3**.** C) Genes that were expressed significantly different upon PCA were exemplarily chosen to visualize the presence and functional states of total T cell (CD3e), cytotoxic T cell (CD8A) with their effector protease granzyme B (GZMB) and regulatory T cells (FOXP3) with their activation marker LRRC32. The sphingosine-1-phosphate receptor 1 (S1PR1) is involved in lymphocytes trafficking and downregulated upon T cell activation in inflamed tissue. Horizontal bars represent the median and error bars the interquartile range. *$p<0.05$,

**p<0.01, and ***p<0.001, n.s.: not significant p≥0.05 in the Kruskal-Wallis test with Dunn's multiple comparison post hoc test.

Figure 3: Donor specific antibodies in subTCMR.

A) All available blood samples paired to liver biopsies (n=365) in our program were screened for DSA. The frequency of DSA in those blood samples are depicted for the whole program (n=100), subclinical T cell-mediated rejection (subTCMR) and the comparators with no histologic rejection (NHR) and clinical TCMR (cTCMR). #: There was no significant enrichment of specificities in the subTCMR cohort compared to the total cohort (Chi square test). B) The specificities of DSA are depicted for subTCMR and the total biopsy program. C) Main differences of histopathological scores between subTCMR with (DSA+) and without DSA (DSA-) (Mann-Whitney-U test). Minor differences are outlined in Table S1?. *p<0.05, **p<0.01 and ***p<0.001, n.s.: not significant p≥0.05.

Figure 4: Upregulation of genes mostly associated with rejection and T cell exhaustion in DSA positive subTCMR.

Analysis of intrahepatic gene expression of 93 markers for rejection (RM), endothelial cells (ECM), immunoregulation (IM), T cell exhaustion (TCEM) and operational tolerance (SOTM). A) Principal component analysis (PCA), calculated using –ΔCt values of all 93 measured genes (Table S2), showed a distinct clustering of biopsies with subclinical TCMR with DSA (DSA+ subTCMR; n=8) and those without (DSA- subTCMR; n=13). B) Heat map summarizing the genes with significantly different expression upon PCA in DSA+ and DSA- subTCMR (p<0.033; FDR<0.01). Transcripts are also listed in Table S4. C) Heat map summarizing the genes with significantly different expression upon PCA in no histologic rejection (green), clinical

TCMR (red), DSA- subTCMR (yellow) and DSA+ subTCMR (blue) ($p<0.05$; FDR<0.05) in a non-supervised analysis. Transcripts are also listed in Table S5. NHR: no histologic rejection; cTCMR: clinical T cell-mediated rejection.