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Title:

Molecular mismatch predicts T cell mediated rejection and *de novo* donor specific antibody formation after living-donor liver transplantation

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Key words:

HLA, eplet mismatch analysis, PIRCHE-II, FCM, MLR

Footnote Page

Abbreviations:

ABO-C, ABO-blood-type compatible; ABO-I, ABO-blood-type incompatible; ABMR, antibody-mediated rejection; CFSE, carboxyfluorescein diacetate succinimidyl ester; CIT; cold ischemic time; CSR, calcineurin inhibitor-sparing immunosuppressive regimen; dnDSA, *de novo* donor specific anti-human leukocyte antigen antibody; DSA, donor-specific anti-human leukocyte antigen antibody; EpMM, eplet mismatch; FCM, flow cytometry; HLA, human leucocyte antigen; LT, liver transplantation; MELD, model for end-stage liver disease; MLR, mixed lymphocyte reaction; MM, mismatch; MMF, mycophenolate mofetil; MP, methylprednisolone; PBMC, peripheral blood mononuclear cell; PIRCHE-II, predicted indirectly recognizable human leukocyte antigen epitopes; PF, precursor frequencies; rATG, rabbit anti-thymocyte globulin; SI, stimulation index; TAC, tacrolimus; TCMR, T cell-mediated rejection

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Abstract

Human leukocyte antigen (HLA) molecular mismatch (MM) analysis improves the prediction of clinical outcomes in kidney transplantation, compared with prediction via traditional antigen MM. However, it remains unclear whether the level of MM can be used for risk stratification among liver transplant recipients. A retrospective observational study of 45 living-donor liver transplantations was performed to evaluate eplet MM as a risk factor for both T cell-mediated rejection (TCMR) in the first month and *de novo* donor-specific antibody (dnDSA) formation. Nine (20%) patients displayed TCMR. HLA-A, -B, -C, and -DRB1 eplet MM number was not associated with TCMR. By contrast, HLA-DQB1 eplet MM (DQB1-EpMM) number was significantly high in patients with TCMR. The predicted indirectly recognizable human leukocyte antigen epitopes (PIRCHE-II) score for the HLA-DQB1 locus (DQB1-PIRCHE-II) was also significantly higher in the TCMR group than in the No-TCMR group. There was a high probability for TCMR to occur either DQB1-EpMM ≥7 or DQB1-PIRCHE-II ≥13. Pre-transplant mixed lymphocyte reaction analyses indicated that there were no significant differences between the anti-donor T-cell proliferation activities of patients with low-(<7) and high-number (≥7) DQB1-EpMM. However, the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T-cells, used as a cytotoxic activity

marker, was high in DQB1-EpMM ≥7. Moreover, either DQB1-EpMM value ≥9 or DQB1-PIRCHE-II ≥3 were a predictor of dnDSA formation. Thus, molecular MMs analysis may be applied towards tailored immunosuppression based on individual risks.

1. Introduction

Human leukocyte antigen (HLA) matching between a donor and a recipient provides numerous benefits, including fewer rejection episodes, prolonged graft and patient survival, and a reduced risk for sensitization in solid organ transplantation (1, 2). However, in liver transplantation (LT), HLA matching has yielded conflicting results in terms of rejection and patient survival (3). A possible mechanism underlying this phenomenon is the limited range of values that are obtained when using traditional low-resolution or high-resolution antigen mismatches (MMs) (0, 1, or 2 per locus). The HLA Matchmaker algorithm, which is based on the concept of that each HLA molecule is a linear sequence of amino acid triplets, relies on the evaluation of eplets, which are the small three-dimensional structure of amino acid residues that are the essential components of immunogenicity (4-6). Results of HLA epitope matching based on this concept have been reported to be superior to those obtained from more conventional HLA matching modalities, especially in kidney transplantation. For instance, eplet MM (EpMM) is associated with the risk of T cell-mediated rejection (TCMR), *de novo* donor-specific antibody (dnDSA) formation, antibody-mediated rejection (ABMR), and graft loss in kidney transplantation (6-8). As an alternative approach for HLA molecular analysis, the predicted indirectly recognizable human leukocyte antigen epitopes (PIRCHE-

II) algorithm was developed to predict the number of recognizable donor-HLA-derived peptides that can be processed and presented by the recipient's HLA class-II molecules (9, 10). Lachmann et al. reported a moderate correlation between the two algorithms and proposed that the PIRCHE-II score was effective as an independent predictor of dnDSA formation and graft survival following kidney transplantation (11).

Kubal et al. reported that, in deceased-donor LT, donor-recipient EpMM is associated with a risk of dnDSA formation and rejection, while Guiral et al. reported that the number of antibody-verified eplets in the HLA-C locus is an independent factor that affects TCMR following deceased-donor LT (12, 13). However, the precise mechanism underlying this phenomenon remains unclear. Reportedly, compared with deceased-donor LT, living-donor LT presents a unique inflammatory and immunological setting that affects immediate and long-term alloimmune responses (14, 15). To evaluate anti-donor T cell responses, we have performed a mixed lymphocyte reaction (MLR) assay using the intracellular fluorescent dye carboxyfluorescein diacetate succimidyl ester (CFSE)-labeling technique (CFSE-MLR) before and after LT (16-18). The objective of this study was to investigate the efficacy of EpMM as a tool for predicting either TCMR or dnDSA formation and to explore the relationship between EpMM and anti-donor T cell responses based on CFSE-MLR in living-

donor LT.

2. Material and methods

2.1. Patients

Between January 2010 and December 2019, 99 patients underwent living-donor LT at the Hiroshima University Hospital. Of these, 54 patients were excluded from the study because of re-transplantation (n = 1), DSAs at the time of LT (n = 11), or incomplete immune monitoring data caused by a limited volume of stored lymphocytes from donors for *in vitro* MLR assays (n = 42). The remaining 45 patients (39 ABO-blood-type compatible recipients [ABO-C] and 6 ABO-blood-type incompatible recipients [ABO-I]) were enrolled in this study. The following information was collected at the time of the transplantation: age, sex, primary disease, model for end-stage liver disease (MELD) score, Child-Pugh score, relationship, HLA MM, and cold ischemic time (CIT). This study was conducted with informed consent using a protocol approved by the Institutional Review Board of the Hiroshima University Hospital (No. Hi-77).

2.2. HLA typing and anti-HLA antibody testing

HLA (-A, -B, -C, -DRB1, and -DQB1) typing of donors and recipients was performed by xMAP R Technology of Luminex Corp., using PCR-sequence-specific oligonucleotide

(SSO) probes at high resolution. Anti-HLA antibodies of all recipients were analyzed before transplantation and monitored annually following transplantation. Serum samples were examined for IgG antibodies against HLA class I or II using methodologies, including either WAKFlow or LABScreen Mixed. All positive evaluations were re-screened, and DSAs were identified using the LABScreen Single Antigen. Mean fluorescence intensity (MFI) values above 1,000 for DSAs against HLA-A, -B, -C, -DRB1, and -DQB1 at the 4-digit level were considered positive.

2.3. HLA molecular mismatch analysis

The number of EpMMs for HLA class I (HLA-A, -B, and -C) and class II (HLA-DRB1 and -DQB1) was determined using HLA Matchmaker software v2.1. PIRCHE-II scores were calculated separately for HLA-A, -B, -C, -DRB1, and -DQB1 by locus and for total loci using the latest version of the PIRCHE algorithm (v3.3.40).

2.4. Desensitization protocol and immunosuppressive regimen

Preoperative desensitization was performed for ABO-I cases. Two weeks before transplantation, a single dose of rituximab (375 mg/m^2 body surface) was administered to the

patients. Subsequently, all subjects received tacrolimus (TAC, target trough level: 5–10 ng/mL) and mycophenolate mofetil (MMF, 10–20 mg/kg/day) and underwent 0–5 sessions of plasmapheresis to decrease anti-blood group isoagglutinin titers, at least by 16-fold, before surgery.

2.5. Immune monitoring via *in vitro* mixed lymphocyte reaction assays

To evaluate the anti-donor immune reactivity of patients, T cell responses to alloantigens were evaluated via MLR assays using an intracellular CFSE labeling technique before and after LT. In brief, peripheral blood mononuclear cells (PBMCs), obtained from the recipients (autologous control), donors, and healthy volunteers (third party control), were prepared as stimulator cells and irradiated with 30 Gy. Responder cells from the recipients were labeled with CFSE (Molecular Probes, Eugene, OR, USA). Both the stimulator and responder cells were adjusted to 2×10^6 cells/mL in AIM-V medium (Invitrogen, NY, USA) and co-cultured in a total volume of 2 mL of medium in 24-well flat-bottom plates (BD Labware, Franklin Lakes, NJ, USA) and incubated at 37 °C in a 5% CO_2 incubator in the dark. Following 5 days of incubation, non-adherent cells were harvested and stained with either phycoerythrin-conjugated CD4 or CD8 monoclonal antibodies (mAbs; BD Pharmingen, San Diego, CA,

USA) together with allophycocyanin-conjugated CD25 mAb (BD Pharmingen). The cells were subjected to multiparameter flow cytometric (FCM) analysis, and dead cells were excluded from the analysis by light scatter and/or propidium iodide fluorescence. $CD4^+$ and $CD8^+$ T cells were selected by gating and analyzed for CFSE fluorescence intensity. Stimulation indices (SIs) were calculated as described previously. To evaluate the immune reactivity of recipients, CFSE-MLR assays were performed at 1, 2, 3 weeks, 1, 3, 6, 12 months and annually after LT.

2.6. Immunosuppressive regimen

The basic immunosuppressive regimen following LT comprised TAC and methylprednisolone (MP) with a gradual tapering of doses. Patients with moderate to severe renal insufficiency (RI) received a calcineurin inhibitor-sparing immunosuppressive regimen (CSR) comprising reduced doses of TAC, MP, and MMF (17). In the conventional regimen, the trough whole blood levels of TAC were maintained between 8 and 15 ng/mL for the first few postoperative weeks and between 5 and 10 ng/mL thereafter. In the CSR, the trough whole blood levels of TAC were maintained between 5 and 10 ng/mL during the first few postoperative weeks and between 3 and 5 ng/mL thereafter.

Based on the proliferation analysis of $CD4^+$ and $CD8^+$ T cell subsets in response to anti-donor versus anti-third party stimuli in protocoled MLR, we categorized the immune status as hypo-, normo-, or hyperresponsive (19). Therapeutic adjustments for immunosuppressants were determined by tapering dosages in cases exhibiting anti-donor hyporesponsiveness in both T-cell subsets and by increasing them for anti-donor hyperresponsiveness.

2.7. Definition and treatment of T cell-mediated rejection

Liver function tests were monitored every day until 2 post-operative weeks and at least every other day until 4 post-operative weeks. TCMR was defined as graft dysfunction, as evidenced by elevated transaminase and/or bilirubin levels, with persistent initial elevation by at least 3 times the upper normal limit in the absence of vascular or biliary complications or infection. Vascular and biliary complications were identified using Doppler ultrasonography. The clinical suspicion of TCMR was supported by the protocoled MLR assay, which can rigorously monitor rejection (16-18). Episodes of rejection were initially treated with either mini pulse (125-250 mg intravenous MP for 2-3 days or more) or with steroid pulse (500 mg intravenous MP for 3 days or more), according to the clinical severity of TCMR, with a gradual tapering of the dose and return to the previous oral double-drug regimen. Rejection

was considered steroid-resistance acute rejection (SRAR) when liver function tests improved by <50% of the highest values after three steroid boluses. Most cases of SRAR were treated with rabbit anti-thymocyte globulin (rATG) (Thymoglobulin; Sanofi K.K., Shinjuku, Japan).

2.8. Statistical analysis

Statistical analyses were performed using JMP version 15.1.0 (SAS Institute, Cary, NC, USA). Quantitative variables were expressed as mean ± standard deviation or as median and range. Student's t-test, Wilcoxon-Mann-Whitney test, chi-squared test, and Fischer's exact test were used to compare variables between the two groups. Kaplan-Meier analysis was used to compare time-to-event variables. Differences between the curves were examined using the log-rank test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Characteristics of patients with or without T cell-mediated rejection episodes

Nine patients (20%) experienced TCMR within 1 month after LT. Five of nine patients were SRAR. Diagnosis of TCMR was also confirmed by pathologic examination or CFSE-MLR results (Supplemental Figure 1). Demographic and immunologic characteristics of the patients that were stratified according to TCMR episodes are shown in Table 1. Regarding the demographic variables, recipient age in the TCMR group was significantly younger than that in the No-TCMR group. Other demographic variables including gender, primary diseases, MELD score, Child-Pugh score, relationship, ABO-blood type compatibility, and CIT were comparable between the two groups.

With respect to the immunologic variables, there was no significant difference in the degrees of HLA allele MM, at the HLA-A, -B, -C, -DRB1, or -DQB1 loci between the two groups (Table 1). The numbers of EpMMs at the HLA-A, -B, -C, and -DRB1 loci were also comparable between the two groups. However, the number of EpMMs at the HLA-DQB1 locus (DQB1-EpMM) was significantly higher in the TCMR group than in the No-TCMR group (Supplemental Figure 2).

The total PIRCHE-II scores were comparable between the two groups. However, PIRCHE-

II score for the HLA-DQB1 locus (DQB1-PIRCHE-II) was significantly higher in the TCMR group than in the No-TCMR group (Table 1).

The mean CNI trough levels at 2 weeks after LT of the patients with TCMR were significantly higher than those without TCMR, perhaps reflecting the results of rejection therapy (Supplemental Figure 3).

3.2. Relationship between the number of molecular MMs at the HLA-DQB1 locus and T cell-mediated rejection

A receiver operating characteristic (ROC) analysis indicated that a DQB1-EpMM value ≥7 would predict TCMR, with a 77.8% sensitivity and 75.0% specificity (area under the curve [AUC] of 0.79). TCMR-free survival stratified by the value of DQB1-EpMM is shown in Figure 1A. Patients with a DQB1-EpMM ≥7 had a significantly higher incidence of TCMR than those with a DQB1-EpMM <7. We have also compared the characteristics of DQB1-EpMM ≥7 patients with or without TCMR episodes. There were no significant differences in demographic variables except for recipient age and the trough levels between the groups (Supplemental Table 1 and Supplemental Figure 4).

ROC analysis indicated that a DQB1-PIRCHE value ≥16 would predict TCMR, with a

55.6% sensitivity and **91.7%** specificity (area under the curve [AUC] of 0.77). TCMR-free survival stratified by the value of DQB1-EpMM is shown in Figure 1B. Patients with a DQB1-PIRCHE ≥16 had a significantly higher incidence of TCMR than those with a DQB1-PIRCHE <16.

To further investigate the relationship between DQB1-EpMM and TCMR, pre-transplant anti-donor T cell responses were analyzed by evaluating the number of DQB1-EpMM using CFSE-MLR assays. There were no significant differences between the anti-donor SI values for both $CD4^+$ and $CD8^+$ T cells of patients with low DQB1-EpMM (<7) and high DQB1-EpMM (≥7). However, the proportion of CD25 expression observed on proliferating anti-donor $CD8^+$ T cells, used as a cytotoxic activity marker, was significantly higher in patients with DQB1-EpMM ≥7 than in patients with DQB1-EpMM <7 (Figure 2). There were no significant differences in MLR results between patients with low DQB1-PIRCHE (<16) and high DQB1-PIRCHE (≥16) (Supplemental Figure 5). During the first month following transplantation, there were no significant differences between the anti-donor SI values for both CD4+ and CD8+ T cell, or the proportions of CD25 expression on proliferating anti-donor CD8+ T cells, of patients with low and high numbers of DQB1-EpMM (Figure 3).

3.3. Relationship between the number of molecular MMs at the HLA-DQB1 locus and *de novo* DSA formation

Nine out of 45 patients (20%) developed dnDSAs during the observation period. Among these 9 patients, Class I dnDSAs were identified in 1 (11.1%) patient, while class II dnDSAs were identified in 8 (88.8%) patients. Demographic variables were comparable between patients with and without dnDSA formation (Table 2). With respect to the immunologic variables, there were no significant differences in the degrees of HLA allele MM at the HLA-A, -B, -C, and -DRB1 loci between the two groups. The number of eplet MMs at the HLA-A, -B, -C, and -DRB1 loci were also comparable between the two groups. However, the degrees of HLA-DQB1 allele MMs and the number of DQB1-EpMM were both significantly higher in patients with dnDSA formation than in patients without dnDSA formation (Supplemental Figure 6). The total PIRCHE-II scores were comparable between the two groups. However, the number of DQB1-PIRCHE-II was significantly higher in the dnDSA group than in the No-dnDSA group (Table 2).

ROC analysis which was performed to investigate the value of DQB1-EpMM that predicts dnDSA formation revealed a DQB1-EpMM value ≥9 with 55.6% sensitivity and 86.1% specificity (AUC of 0.76). Furthermore, dnDSA-free survival stratified by the DQB1-EpMM

value is shown in Figure 4A. Patients with DQB1-EpMM ≥9 had a significantly higher incidence of dnDSA formation directed against all HLA loci than those with DQB1-EpMM <9. Patients with DQB1-EpMM ≥9 had also a significantly higher incidence of dnDSA formation directed against HLA-DQB1 than those with DQB1-EpMM <9 (Supplemental Figure 7). ROC analysis indicated that a DQB1-PIRCHE value ≥3 would predict dnDSA, with a **100.0%** sensitivity and **47.2%** specificity (area under the curve [AUC] of 0.75). dnDSA-free survival stratified by the value of DQB1-PIRCHE is shown in Figure 4B. Patients with a DQB1-PIRCHE ≥3 had a significantly higher incidence of dnDSA formation than those with a DQB1-PIRCHE <3.

There was no significant difference in mean CNI trough levels at 3 months, 1 year and 3 years after LT between the patients with and without dnDSA. However, the mean tough levels at 5 years after LT in patients with dnDSA were significantly lower those without dnDSA (Supplemental Figure 8). There was no significant difference in mean trough levels between DQB1-EpMM <9 and DQB1-EpMM ≥9 (Supplemental Figure 9).

We did not observe any associations between dnDSA formation and clinical outcomes such as TCMR or graft loss. During five years following transplantation, anti-donor SI values for both $CD4^+$ and $CD8^+$ T cells, as well as the proportion of CD25 expression on proliferating

anti-donor $CD8^+$ T cells, tended to be higher in the patients with DQB1-EpMM ≥9 than in patients with DQB1-EpMM <9 (Figure 5). There were no significant differences in MLR results between patients with low DQB1-PIRCHE (<3) and high DQB1-PIRCHE (≥3) (Supplemental Figure 10).

4. Discussion

Liver allograft rejection remains a significant cause of morbidity and graft failure in liver transplant recipients (15). TCMR, also previously known as “acute cellular rejection”, occurs most commonly within the first month following LT, where its prevalence may vary from 20% to 50% depending on the definition used (14). Various factors such as deceased donors, CIT, younger recipients, and recipients with primary biliary cirrhosis, or hepatitis C have been reported as risk factors for TCMR (15, 20). In this study, younger recipients were consistently observed as risk factors for TCMR. Because the magnitude of allo-responses decreases with increasing age, older recipients may be associated with a reduced incidence of rejection (21). As opposed to previous reports, we did not observe CIT as a risk factor, presumably because of the short ischemic times in our living-donor LT cohort.

There was no significant difference between the degrees of HLA alleles MM in patients with TCMR and those without. However, in HLA molecular analysis, the number of DQB1-EpMM and DQB1-PIRCHE were significantly higher in the TCMR group than in the No-TCMR group. It has been recently reported that the number of class I eplet MMs is associated with TCMR (12, 13). The fact that our findings contradict the above findings may be because of the use of smaller sample sizes, racial/ethnic homogeneity, and a better HLA-matched

living-donor LT cohort.

In this study, the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells estimated via a pre-transplant CFSE-MLR assay was significantly higher in patients with DQB1-EpMM ≥7 than in patients with DQB1-EpMM <7. We have previously indicated that a notable elevation of CD25 expression on proliferating $CD8^+$ T cells reflects their cytotoxic activity towards donor cells (16), and may be applied to algorithms to determine anti-donor alloreactivity in LT patients (19). Early TCMR is attributable to direct alloantigen presentation, which may be precisely evaluated via a CFSE-MLR assay. Thus, pre-transplant evaluation of anti-donor T cell responses based on eplet MMs may be useful for tailoring immunosuppression according to individual risks.

Reportedly, dnDSA formation following LT is an independent risk factor for patient death and graft loss (22, 23). The risk factors previously reported for dnDSA formation include young recipient age, a low MELD score, inadequate immunosuppression, and HLA-DQ allele MMs (22). Recently, Kubal et al. reported that the number of class II MM eplets estimated via the HLAMatchmaker algorithm was strongly associated with the risk of class II dnDSA formation following decease donor LT (12), and more recently, PIRCHE-II algorithms have been used as effective tools for predicting dnDSA formation following LT

(24). Consistent with previous reports, the degrees of HLA-DQB1 allele MM and the number of DQB1-EpMM were both significantly higher in patients with dnDSA formation than in patients without.

A serial CFSE-MLR assay was performed to evaluate the status of T cell immunity in recipients. During the early phases following surgery, individual variations in SIs of anti-donor $CD4^+$ and $CD8^+$ T cells in patients with a high DQB1-EpMM number were generally larger than those in patients with a low DQB1-EpMM number, indicating that patients with a high DQB1-EpMM number displayed a higher incidence of TCMR within the first month than patients with a low DQB1-EpMM number. Notably, regardless of CFSE-MLR-based optimization of immunosuppressive therapy, anti-donor SI values for $CD4^+$ T cells in patients with a high number of DQB1-EpMM tended to be higher than those in patients with a low number of DQB1-EpMM. This may lead to a higher incidence of dnDSA formation in patients with a high number of DQB1-EpMMs. However, dnDSA formation does not necessarily result in either ABMR or graft loss. Further analyses that included these data may potentially provide more convincing evidence pertaining to the clinical benefits of epitope analysis.

Our study has several limitations. This retrospective study features a relatively small sample

size, brief observation period relatively high MELD scores, racial/ethnic homogeneity, and a better HLA-matched living-donor LT cohort. Despite the above-mentioned limitations, this study, to our knowledge, is the first to reveal the relationship between eplet MM numbers and anti-donor T cell responses in LT recipients. We believe that these observations will be important for tailoring immunosuppression to suit individual risks.

In conclusion, the number of molecular MMs may predict both TCMR and dnDSA formation. Thus, molecular MMs analysis may be applied to tailor immunosuppression according to individual risks. A rigorously designed, large-scale prospective study may be needed to validate these conclusions.

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Figure legends

Figure 1. Kaplan-Meier T cell-mediated rejection-free survival curves stratified by the number of molecular mismatches at the HLA-DQB1 locus

(A) T cell-mediated rejection (TCMR)-free survival within 1 month after liver transplantation stratified by EpMM at the HLA-DQB1 locus (DQB1-EpMM) with a cutoff value of 7. Gray line, DQB1-EpMM <7; black line, DQB1-EpMM ≥7. (B) TCMR-free survival within 1 month after liver transplantation stratified by PIRCHE-II score for the HLA-DQB1 locus (DQB1-PIRCHE) with a cutoff value of 16. Gray line, DQB1-PIRCHE <16; black line, DQB1-PIRCHE ≥16.

Figure 2. Association between the number of DQB1-EpMMs and pre-transplant anti-donor T cell responses based on CFSE-MLR assays

Pre-transplant anti-donor T cell responses based on CFSE-MLR were analyzed by the number of EpMMs at the HLA-DQB1 locus (DQB1-EpMM). The stimulation index (SI) values for anti-donor $CD4^+$ T cells (A) and $CD8^+$ T cells (B), and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells (C) are shown. White box, DQB1-EpMM <7 (n = 29); gray box, DQB1-EpMM ≥7 (n = 16). Data are shown as median, 25th and 75th

percentiles, and range. The Wilcoxon-Mann-Whitney test was used to test the differences between DQB1-EpMM <7 and DQB1-EpMM ≥7. * = P < 0.01.

Figure 3. Kinetics of stimulation index (SI) and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells in patients with low and high numbers of DQB1-EpMMs during the first month after liver transplantation.

Post-transplant anti-donor T cell responses based on CFSE-MLR were analyzed by the number of EpMMs at the HLA-DQB1 locus (DQB1-EpMM). The SI values for anti-donor $CD4^+$ T cells (A) and $CD8^+$ T cells (B), and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells (C) are shown. White box, DQB1-EpMM <7 (n = 29); gray box, DQB1-EpMM ≥7 (n = 16). Data are shown as median, 25th and 75th percentiles, and range. The Wilcoxon-Mann-Whitney test was used to test the differences between DQB1-EpMM <7 and DQB1-EpMM ≥7.

Figure 4. Kaplan-Meier *de novo* DSA-free survival curves stratified by the number of molecular mismatches at the HLA-DQB1 locus

(A) *de novo* DSA (dnDSA)-free survival stratified by EpMM at the HLA-DQB1 locus

(DQB1-EpMM) with a cutoff value of 9. Gray line, DQB1-EpMM <9; black line, DQB1-EpMM ≥9. (B) dnDSA-free survival stratified by PIRCHE-II score for the HLA-DQB1 locus (DQB1-PIRCHE) with a cutoff value of 3. Gray line, DQB1-PIRCHE <3; black line, DQB1-PIRCHE ≥3.

Figure 5. Stimulation index (SI) kinetics and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells in patients with low and high numbers of DQB1-EpMM during the first year after liver transplantation.

Post-transplant anti-donor T cell responses based on CFSE-MLR were analyzed via the number of EpMMs at the HLA-DQB1 locus (DQB1-EpMM). The SI values for anti-donor $CD4^+$ T cells (A) and $CD8^+$ T cells (B), and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells (C) are shown. White box, DQB1-EpMM <9 (n = 35); gray box, DQB1-EpMM ≥9 (n = 10). Data are shown as median, 25th and 75th percentiles, and range. The Wilcoxon-Mann-Whitney test was used to test the differences between DQB1-EpMM <9 and DQB1-EpMM ≥9. *= $P < 0.01$.

Supplemental Figure 1. Representative histology and its CFSE-MLR result in TCMR case

Supplemental Figure 2. Comparison the number of DQB1-EpMM between the patients with and without TCMR.

Supplemental Figure 3. Kinetics of the CNI trough levels in patients with and without TCMR during the first month after liver transplantation.

White box, patients without TCMR (n = 36); gray box, patients with TCMR (n = 9).

Supplemental Figure 4. Kinetics of the CNI trough levels in DQB1-EpMM ≥7 patients with and without TCMR during the first month after liver transplantation.

White box, patients without TCMR (n = 9); gray box, patients with TCMR (n = 7).

Supplemental Figure 5. Association between the number of DQB1-PIRCHE and pre-transplant anti-donor T cell responses based on CFSE-MLR assays.

Pre-transplant anti-donor T cell responses based on CFSE-MLR were analyzed by PIRCHE-

II score for the HLA-DQB1 locus (DQB1-PIRCHE). The stimulation index (SI) values for anti-donor $CD4^+$ T cells (A) and $CD8^+$ T cells (B), and the proportion of CD25 expression on proliferating anti-donor $CD8^+$ T cells (C) are shown. White box, DQB1-PIRCHE <16 (n = 38); gray box, DQB1-PIRCHE ≥16 (n = 7). Data are shown as median, 25th and 75th percentiles, and range. The Wilcoxon-Mann-Whitney test was used to test the differences between DQB1-PIRCHE <16 and DQB1-PIRCHE≥16.

Supplemental Figure 6. Comparison the number of DQB1-EpMM between the patients with and without dnDSA.

Supplemental Figure 7. Kaplan-Meier de novo DSA directed against HLA-DQB1 -free survival curves stratified by the number of eplet mismatches at the HLA-DQB1 locus.

de novo DSA directed against HLA-DQB1-free survival stratified by EpMM at the HLA-DQB1 locus (DQB1-EpMM) with a cutoff value of 9. Gray line, DQB1-EpMM <9; black line, DQB1-EpMM ≥9.

Supplemental Figure 8. Kinetics of the CNI trough levels in patients with and without

dnDSA during the five years after liver transplantation.

White box, patients without dnDSA (n = 36); gray box, patients with dnDSA (n = 9).

Supplemental Figure 9. Kinetics of the CNI trough levels in patients with low and high numbers of DQB1-EpMMs during the five years after liver transplantation.

White box, DQB1-EpMM <9 (n = 35); gray box, DQB1-EpMM ≥9 (n = 10).

Supplemental Figure 10. Stimulation index (SI) kinetics and the proportion of CD25 expression on proliferating anti-donor CD8+ T cells in patients with low and high numbers of DQB1-PIRCHE during the first year after liver transplantation.

Post-transplant anti-donor T cell responses based on CFSE-MLR were analyzed via PIRCHE-II score for the HLA-DQB1 locus (DQB1-PIRCHE). The SI values for anti-donor CD4+ T cells (A) and CD8+ T cells (B), and the proportion of CD25 expression on proliferating anti-donor CD8+ T cells (C) are shown. White box, DQB1-PIRCHE <3 (n = 17); gray box, DQB1-PIRCHE ≥3 (n = 28). Data are shown as median, 25th and 75th percentiles, and range. The Wilcoxon-Mann-Whitney test was used to test the differences between DQB1-PIRCHE <3 and DQB1-PIRCHE ≥3.