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ORIGINAL ARTICLE

Donor and recipient HLA/KIR genotypes do not predict liver transplantation outcome

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Summary

Whether or not Natural Killer (NK) cells affect the immune response to solid organ allografts is still controversial. Main determinants of NK-cell activation are specific HLA/killer-cell immunoglobulin-like receptors (KIR) interactions that, in transplantation, may induce NK-cell alloreactivity. So far, in liver transplantation (LTX) donor-versus-recipient alloreactivity has not been investigated; in addition, studies of predicted recipient-versus-donor NK-cell alloreactivity have led to contradicting results. We typed a cohort of LTX donors and recipients for HLA-C/Bw4 and KIRs. We estimated the effect of NK-cell alloreactivity, as predicted by classically used models, in the donor-versus-recipient direction. The results indicate that HLA/KIR mismatches in the donor-versus-recipient direction do not predict graft rejection nor graft or patient survival, suggesting that donor-derived NK cells do not play a major role in LTX outcome. In addition, when considering predicted NK-cell alloreactivity in the reverse direction (recipient-versus-donor), we first confirmed that donor HLA-C genotype was not associated with acute rejection, graft or patient survival and secondly we found that none of the models describing NK-cell alloreactivity could predict LTX outcome. Overall our observations suggest that, in contrast to what is shown in haematopoietic stem cell transplantation, donor-derived NK cells may not contribute in preventing liver graft rejection, and that recipient-versus-donor NK-cell alloreactivity does not predict LTX outcome.

Introduction

The role of the adaptive immune response in organ transplantation has been clearly established [1,2], however, the effects of the innate response are far less clear. Natural Killer (NK) cells are part of the innate immunity and can distinguish between self and non self tissues by use of a variety of receptors that recognize specific alleles of MHC class I molecules expressed on cell surfaces. The main receptors involved in self-recognition are the killer-cell immunoglobulin-like receptors (KIRs). By binding specific MHC class I alleles on target cells (Table 1 in reference [3]), KIRs constitute an effective tool detecting both transformed and virally infected cells as well as recognizing 'missing self' in transplantation settings. The most relevant MHC class I alleles linked to NK-cell activity are HLA-A3 and -A11, HLA-Bw4 and HLA-C [3]. HLA-C has recently raised interest as a possible factor involved in prediction of transplantation outcome. Importantly, all of the >250 alleles of HLA-C are recognized by KIRs and can be divided into one of two groups (HLA-C1 and HLA-C2) with regard to their ability to bind KIRs [3].

Table 1. Demographics and other relevant genetic characteristics of the study population (a), causes of graft (b) and patient (c) loss.

(a)

	n = 348	Graft survival*	Patient survival†	Acute rejection
Number of events		Graft loss = 41/348	Patient loss = 71/348	Acute rejection = 81/348
Characteristics	No. (range or percentage)	*P*-value‡	*P*-value‡	*P*-value‡
Recipient age	47.1 (16–69)	**<0.001**	**<0.001**	–
Recipient gender M:F	203:145	0.53	0.16	**0.02**
Donor age	41.7 (11–72)	**0.009**	0.83	–
Donor gender M:F	170:178	0.26	0.46	0.69
Ethnicity				
Caucasian	300 (86.2)	0.11	0.12	0.08
Black	29 (8.3)			
Asian	19 (5.5)			
Gender mismatch no mismatch:mismatch	191:157	**0.04**	0.21	0.16
Diagnosis				
Viral hepatitis	86 (24.7)	0.16	0.45	**<0.001**
Auto-immune aetiology¶	100 (28.7)			
Alcohol abuse	39 (11.2)			
Acute fulminant hepatitis	27 (7.8)			
All other causes	96 (27.6)			

Statistically significant associations are indicated in bold.
*Median graft survival time was of 7.4 ± 5.2 years (range 0–22.1).
†Median patient survival time was of 7.8 ± 5.0 years (range 0–22.1).
‡*P*-values indicate a univariate association of the specific factor with graft survival, patient survival or acute rejection.
¶Auto-immune aetiology includes: primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and auto-immune hepatitis (AIH).

(b)

Causes of graft loss (graft-related death or re-LTX) (*n* = 41)	*n* (%)
Ischaemic type biliary lesions	20 (48.8)
Chronic rejection	8 (19.5)
Recurrence of original disease (HCV, PSC, AIH)	8 (19.5)
Vascular complications	4 (9.8)
Recurrence of HCC	1 (2.4)

(c)

Causes of patient loss (*n* = 71)	*n* (%)
Not graft related	40 (56.4)
Ischaetype biliary lesions	2 (2.8)
Chronic rejection	4 (5.6)
Recurrence of disease (HCV, PSC, AIH)	5 (7.0)
Vascular complications	1 (1.4)
Recurrence of HCC	9 (12.7)
De novo tumour	10 (14.1)

re-LTX, re-liver transplantation; HCV, hepatitis C virus; PSC, primary sclerosing cholangitis; AIH, auto-immune hepatitis; HCC, hepatocellular carcinoma.

Results from animal models did not indicate a role for NK cells in solid organ transplantation [4–6]. Nevertheless, recent studies introduced the novel concept that recipient NK cells participate in both acute and chronic rejection after solid organ transplantation by modulating the host immune response rather than directly affecting the transplanted organ [6–11]. Importantly, a new perspective in the analysis of the role of NK cells in transplantation has been introduced by studies in haematopoietic stem cell transplantation (HSCT) [3,12–14]. In this context, donor-versus-recipient NK-cell alloreactivity prevents graft rejection besides

inducing additional beneficial effects for the recipient [12]. Similar to what was observed in HSCT we hypothesized that donor NK cells may have a protective role in liver transplantation (LTX) on the basis of two main observations. First, donor-versus-recipient alloreactivity has been occasionally observed in the context of LTX, since cases of graft-versus-host disease have been recorded [15,16]. Secondly, a previous publication from our group [17] has shown that highly cytotoxic donor NK cells derived from the graft are consistently transferred into the recipient upon LTX.

With regard to the effect of recipient-versus-donor NK-cell alloreactivity only few studies have addressed this issue in solid organ transplantation in humans [18,19]. In kidney transplantation missing HLA-C or HLA-Bw4 KIR-ligands in the recipient-versus-donor direction has no impact on either acute rejection [20,21] or graft survival [22]. Importantly, contradictory results have been published so far for LTX. In one study, HLA-C disparity between recipient and donor was found to be correlated with a higher risk of acute rejection of the liver graft [23], but this was not confirmed by other groups [24–26]. Moreover, while Hanvesakul *et al.* [24] provided evidence that the presence of HLA-C2 in the donor was associated with improved long-term graft and patient survival after LTX, Tran *et al.* [27] found no impact of donor HLA-C2 genotype on 10-years graft or patient survival. Therefore, the effect of HLA/KIR genotypes and recipient NK cells on liver graft rejection and survival is still an unresolved issue.

In the present study, we explored for the first time, the effect of donor-versus-recipient NK-cell alloreactivity on LTX outcome. For this purpose, we used common models describing NK-cell alloreactivity to estimate the effects of donor NK cells on acute rejection, graft and patient survival. In addition, by analysing our study cohort in terms of recipient-versus-donor alloreactivity, we aimed at adding new evidence to the existing data on the effects of HLA/KIR matching on LTX outcome.

Patients and methods

Patients

This study includes 348 LTX performed at the Erasmus Medical Center in Rotterdam (The Netherlands) between 1987 and 2008. All LTX were first grafts, while re-transplantations or multi-organ transplantations were excluded. Only patients with graft survival of >7 days were included. From 322 paired donors and recipients HLA-Bw4 typing was available. From 260 pairs DNA of both donor and recipient was archived, and used for typing of HLA-C. A smaller cohort of 153 donor/recipient pairs was typed for the KIR genes (Fig. 1). To exclude

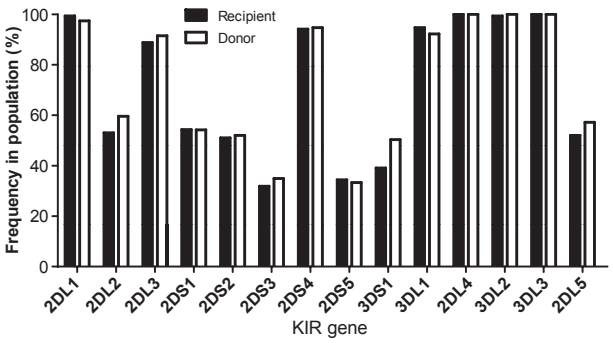

Figure 1 KIR gene distribution in our cohort of liver transplant patients. KIR genotyping was performed on 153 recipients (black bars) and their paired donors (white bars). The KIR genes are indicated on the *x*-axis while the *y*-axis reports the frequencies for each donor and recipient KIR.

that partial typing of the cohort had determined a selective exclusion of donors or recipients we compared the baseline characteristics of the groups with complete or non complete typing and verified that they did not differ in terms of age and gender. The Ethical Committee of the Erasmus MC approved the study.

Clinical data

All clinical data considered as endpoints (e.g. graft failure, acute/chronic rejection, biliary complications) were re-evaluated for each case by an experienced transplant hepatologist (HJM). Acute rejection was defined as an episode with increased liver enzymes together with histopathological evidence (Rejection Activity Index-score ≥5) combined with a biochemical response to the treatment by high dose corticosteroids or another change in immunosuppressive treatment. Graft loss was defined as graft-related patient death or graft failure requiring re-transplantation. For the analysis of patient survival, patient loss was defined as patient death for any cause. Ischaemic bile duct damage was defined as the development of diffuse intra- and/or extra-hepatic biliary strictures and dilatations in the presence of normal hepatic arterial circulation and in the absence of other diagnosis such as recurrence of primary sclerosing cholangitis. Chronic rejection was defined as deteriorating liver graft functions accompanied by loss of small bile ducts in 50% or more of the portal tracts in sequential needle biopsy specimens.

Standard immunosuppression included a combination of calcineurin inhibitors (either cyclosporine or tacrolimus) and steroids, supplemented with either azathioprine or induction therapy with an anti-IL-2 receptor blocking antibody.

HLA and KIR genotyping

The presence of HLA-Bw4 was deducted from the serological typing routinely performed before LTX. For HLA-C and KIR genotyping, DNA was extracted by the classical salting out method. Molecular typing for HLA-C was performed by polymerase chain reaction with sequence-specific oligonucleotide probes using LABType® SSO C Locus kit (One-Lambda, Canoga Park, USA) according to ASHI standards. Differentiation into HLA-C1/C2 was made on the basis of Lysine or Asparagine at position 80 of HLA-C. Inhibitory and activating KIRs (14 in total; Fig. 1) were typed in donor and recipient samples using sequence specific primers according to the protocol previously described [28].

Models predicting NK-cell alloreactivity

Predictions of NK-cell alloreactivity were initially performed on the basis of two main models: 'missing self' and 'missing ligand' model. The definition of the 'missing self' model was based on two recent studies [22,29]. Briefly, for prediction of NK-cell alloreactivity in donor-versus-recipient direction, donor/recipient combinations were divided into three groups: (i) 'C1/2–Bw4 matched' group, if donor and recipient shared the same HLA-C1, C2 and Bw4 epitopes, (ii) 'C1/2–Bw4 mismatched compatible' group, if donor and recipient were mismatched for C1, C2 or Bw4 epitopes, but the donor's KIR ligands were not missing in the recipient's HLA genotype, (iii) 'C1/2–Bw4 mismatched incompatible' group, if the recipient's HLA genotype did not include a C1, C2 or Bw4 epitope that was present in the donor.

The 'missing ligand' model in donor-versus-recipient direction states that for each inhibitory KIR expressed in the donor, its ligand needs to be present in the recipient so as to avoid NK-cell alloreactivity. Missing ligand combinations were defined positive when the recipient was missing at least one of the KIR ligands for which the donor had a KIR [13,14]. Following previous observations [30,31], 'unlicensed NK cells', meaning donor NK cells that possess a certain KIR for which the corresponding ligand is not present in the donor genotype, were considered as potentially able to be activated by the absence of their ligand in the recipient.

In addition to the previous analysis, we considered two other models, both named 'strength of inhibition': the first including only inhibitory KIRs and the second including both inhibitory and activating KIRs. As the activation of NK cells is known to be the net result of signals from activating and inhibitory KIRs we included the donor KIR-gene repertoire as to predict NK-cell alloreactivity. As for the inhibitory KIRs, there is a hierarchy in the strength of inhibition that defines how the combination of KIR2DL1 with HLA-C2 leads to the strongest NK-cell inhibition, whereas KIR2DL2 with HLA-C1 gives an intermediate inhibition and KIR2DL3 with HLA-C1 confers the least inhibition [20, 32]. To perform this analysis (strength of NK inhibition, predicted by donor inhibitory KIRs and recipient HLA) we classified donor/recipient pairs according to the three above mentioned categories of strength of inhibition and we determined their association with graft survival, patient survival and acute rejection. Next, in the final model we aimed at testing the combined effect of inhibitory and activating donor KIRs, and recipient HLA genotype, on LTX outcome. With regard to the activating receptors, two groups of KIR-haplotypes can be distinguished: while most stimulatory KIR genes are present in the haplotype B, the haplotype A contains only one stimulatory receptor (KIR2DS4) [33]. Accordingly, individuals carrying the AA KIR genotype will exhibit a lower NK-cell activation originating from activating KIRs compared to individuals with the AB or BB KIR genotypes [18,34]. To perform the analysis of the combined effect of inhibitory and activating KIRs we categorized the donor/recipient pairs on basis of the combination of their strength of inhibition (determined by inhibitory KIRs) and the KIR haplotype (determined by activating KIRs). Donor/recipient pairs were therefore divided in six possible combinations, corresponding to different degrees of inhibition, e.g. in case of donor-versus-recipient alloreactivity, the combination with the strongest inhibition was given by a homozygous HLA-C2/C2 recipient, with a donor possessing KIR2DL1 and AA haplotype.

Statistical analysis

Survival data were analysed using the Kaplan–Meier method and the log-rank test. Multivariate Cox regression was used to verify that single factors were independently associated with graft or patient survival. Crosstabs were used for all correlations with acute rejection and were tested by use of the Pearson chi-square test. Probability (P) values of less than 0.05 were considered significant. All statistical analyses were performed using SPSS (version 17.0.2, Chicago, IL, USA).

Results

Donor-versus-recipient NK-cell alloreactivity, as predicted by the missing self model, does not affect LTX outcome

Patients' demographics and their associations with graft survival, patient survival and acute rejection are reported in Table 1a, whereas causes of graft and patient loss are listed in Table 1b and 1c.

We herein explored the effect of hepatic donor NK-cell alloreactivity on three LTX outcomes: acute rejection, graft failure and graft survival. For this purpose, we estimated the level of donor NK-cell activation as predicted by a number of classically used models. We first analysed our study cohort by using the 'missing self' model, for both HLA-C and -Bw4, to predict donor-versus-recipient NK cell alloreactivity. The analysis was performed by stratifying donor/recipient pairs into three groups (see Patients and Methods). No differences in terms of graft survival ($P = 0.13$), patient survival ($P = 0.13$) or incidence of acute rejection ($P = 0.71$) were found among the three groups (Table 2a). The two groups 'C1/2-Bw4 matched' and 'C1/2-Bw4 mismatched compatible' represent all cases of KIR-ligand compatible transplants, while the 'C1/2–Bw4 mismatched incompatible' group identifies the KIR-ligand incompatible transplants. This additional classification showed that 'missing self' in donor to recipient direction had no effect on LTX outcome compared to compatible donor/recipient pairs (Table 2a). Likewise, analysis of the 'missing self' model by considering donor-versus-recipient disparities only in the HLA-C epitope or the HLA-Bw4 molecule did not show any effect on LTX outcome (Table 2a).

LTX outcome is not influenced by KIR-ligand mismatching or donor KIR gene repertoire

For this study, we genotyped 153 donor/recipient pairs for 14 different KIR genes. The KIR gene distribution in both donors and recipients is depicted in Fig. 1 and did not differ from the frequencies observed in healthy popu-

Table 2. *P*-values of the univariate analyses performed considering the most relevant models predicting NK-cell alloreactivity (a) and of Cox regression multivariate analysis of all factors univariately associated with liver transplantation outcomes (b).

(a)

	Donor versus recipient reactivity			Recipient versus donor reactivity		
	Graft survival	Patient survival	Acute rejection	Graft survival	Patient survival	Acute rejection
Model	*P*-value	*P*-value	*P*-value	*P*-value	*P*-value	*P*-value
Missing self HLA-C and -Bw4 (three categories)	0.13	0.13	0.71	0.69	0.86	0.49
Missing self – compatible LTX versus incompatible LTX*	0.21	0.13	0.61	0.42	0.58	0.28
Missing self – only HLA-C	0.65	0.54	0.87	0.97	0.64	0.97
Missing self – only HLA-Bw4	0.85	0.46	0.85	**0.01**	0.69	0.51
Missing ligand	0.48	0.12	0.79	0.11	0.76	0.75
Strength of NK inhibition (inhibitory KIRs)	0.47	0.97	0.51	0.75	0.49	0.61
Strength of NK inhibition (inhibitory and activating KIRs)	0.57	0.49	0.58	0.49	0.21	0.79
Presence of donor HLA-C2	0.48	0.64	0.61	0.06	0.18	0.66
Donor HLA-C genotype	0.63	0.88	0.26	**0.05**	**0.01**	0.79

Statistically significant associations are indicated in bold. Associations with graft or patient survival were analysed by using the Log-rank test. Associations with acute rejection were performed using the Pearson chi-square test.
*Compatible LTX versus incompatible LTX = 'C1/2-Bw4 matched' and 'C1/2-Bw4 mismatched compatible' versus 'C1/2-Bw4 mismatched incompatible'.

(b)

	Significance	Hazard ratio	95% CI
Multivariate analysis for all factors univariately associated with graft survival			
Recipient age (for a 10-year increase in age)	**0.01**	0.97	0.94–0.99
Donor age (for a 10-year increase in age)	**0.02**	1.03	1.00–1.06
Gender mismatch	**0.05**	0.45	0.21–0.99
Missing HLA-Bw4 (REC versus DON)*	0.96	0.00	0.00–8.8E + 254
Donor HLA-C genotype	0.75	1.08	0.67–1.73
Multivariate analysis for all factors univariately associated with patient survival			
Recipient age (for a 10-year increase in age)	**0.03**	1.03	1.00–1.06
Donor HLA-C genotype	0.07	1.48	0.96–2.26

Statistically significant associations are indicated in bold.
*Compared with 'non missing HLA-Bw4' donor/recipient pairs.

lations [21,35]. Analysis of our cohort by use of the 'missing ligand' model (see Patients and Methods) revealed that missing a KIR-ligand in the donor-versus-recipient direction had no effect on LTX outcome (Table 2a).

In addition, analysis of potential donor-versus-recipient NK-cell alloreactivity in terms of hierarchy in strength of inhibition (see Patients and Methods) showed no significant association either with graft or patient survival or with acute rejection (Table 2a). Inclusion of the donors' KIR haplotype (accounting for the number of activating KIRs in the donor genotype) in this model did not result in any significant association with LTX outcome (Table 2a). Altogether our results indicate that none of the current models describing NK-cell alloreactivity in the donor-versus-recipient direction could predict LTX outcome.

Recipient-versus-donor NK-cell alloreactivity

We herein considered the most classical perspective for predicting LTX outcome: NK-cell alloreactivity in the recipient-versus-donor direction. For this purpose, we applied the same set of models outlined above to explore potential NK-cell alloreactivity in the recipient-versus-donor direction. The majority of the models did not show an association with acute rejection, graft or patient survival (Table 2a), suggesting the absence of a major role of recipient-versus-donor NK-cell alloreactivity in LTX outcome. However, analysis of the 'missing self' model by considering the single HLA-Bw4 molecule was significantly associated with better graft survival in univariate analysis ($P = 0.01$; Table 2a). Nevertheless, the effect was completely abrogated in multivariate analysis ($P = 0.69$; Table 2b) when considering all factors univariately associated with graft survival (see next paragraph).

Effect of donor HLA-C genotype on graft and patient survival

We then completed our study by analysing the possible association between LTX outcome and donor HLA-C genotype [homozygous C1C1: 102 (39.2%); heterozygous C1C2: 124 (47.7%); homozygous C2C2: 34 (13.1%)]. In our cohort, the donor HLA-C genotype ($P = 0.05$) or the presence of HLA-C2 in the donor ($P = 0.06$) were not associated with improved graft survival (Fig. 2a, Table 2a). More specifically, the 10-year graft survival was

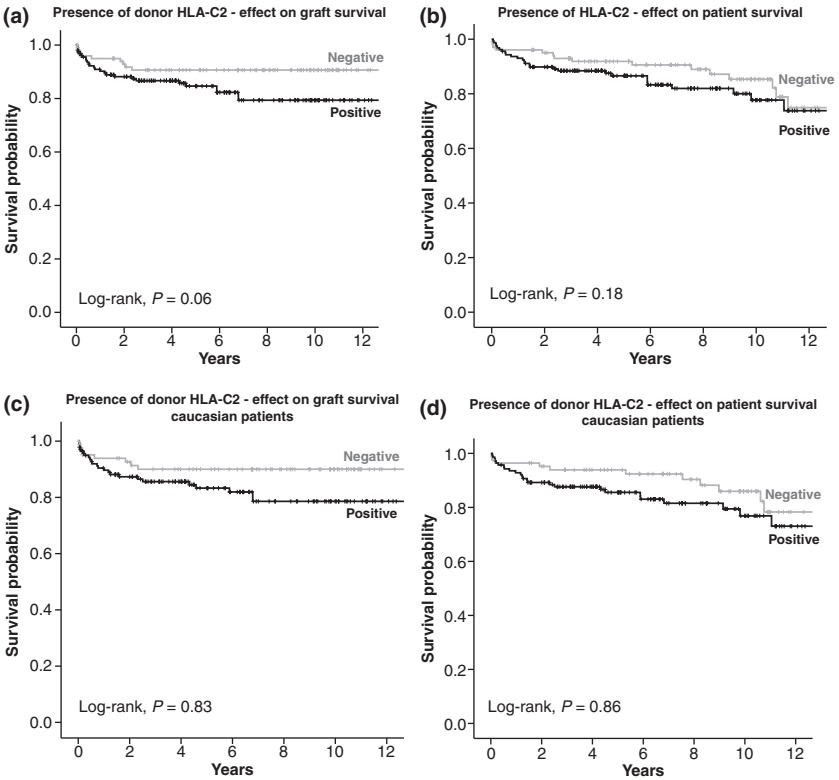

Figure 2 Effect of donor HLA-C2 on graft and patient survival after liver transplantation. Kaplan–Meier survival curves of graft (a, c) and patient (b, d) survival after liver transplantation on basis of the presence (positive) or absence (negative) of HLA-C2 in the donor genotype. (a, b) complete cohort. (c, d) Caucasian patients only.

79.4 ± 3.9% in the presence and 90.7 ± 3.0% in the absence of the donor HLA-C2 allele ($P = 0.06$). Comparing graft survival rates among the three possible donor HLA-C genotypes revealed a trend towards a gene dose effect (overall log rank P-value = 0.05). HLA-C2 homozygous donors led to the lowest graft survival rate (HLA-C2 versus HLA-C1 homozygous donors, $P = 0.02$), whereas transplants from HLA-C1C2 heterozygous donors showed an intermediate rate of graft loss. However, in multivariate analysis this effect was abrogated and only recipient age, donor age and gender mismatch were associated with graft failure (Table 2b).

In univariate analysis, donor HLA-C genotype was significantly associated with patient survival ($P = 0.01$) (Table 2a); conversely, the presence of HLA-C2 in the donor did not affect patient survival ($P = 0.18$) (Fig. 1b, Table 2a). Homozygous HLA-C2 donors showed the lowest patient survival rate (HLA-C2 versus HLA-C1 homozygous donors, $P = 0.007$; homozygous HLA-C2 versus HLA-C1C2 donors, $P = 0.01$). However, in multivariate Cox regression analysis recipient age abrogated the effect of donor HLA-C genotype on patient survival (Table 2b).

To eliminate a possible bias related to the inclusion of multiple ethnicities we repeated the analysis limiting our study group to Caucasian patients typed for HLA-C ($n = 224$). The results indicated a trend similar to the one described for the total cohort (Fig. 2c, d).

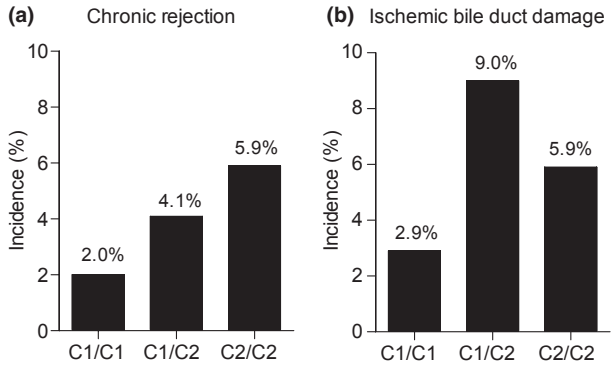

Figure 3 Impact of donor HLA-C genotype on causes of histological features of chronic injury. All cases of chronic rejection (a) and ischaemic bile duct damage (b) (not only associated with graft loss) are grouped on basis of the donor genotype to evaluate a possible effect on hepatic injury. Donor HLA-C genotype is not significantly associated with incidence of chronic rejection ($P = 0.49$) or ischaemic bile duct damage ($P = 0.17$). However, the trend suggests a negative effect of donor HLA-C2 on chronic rejection (C1/C1, $n = 2/102$; C1/C2, $n = 5/122$; C2/C2, $n = 2/34$), whereas no effect could be evidenced on ischaemic bile duct damage (C1/C1, $n = 3/102$; C1/C2, $n = 11/122$; C2/C2, $n = 2/34$; for four patients, diagnosed with ischaemic bile duct damage, donor HLA-C typing was not available). Statistical analysis was performed using Pearson chi-square test.

Donor HLA-C genotype does not correlate with the type of graft injury nor with acute graft rejection

Herein, we analysed the association between the presence of donor HLA-C2 and graft injury. Specifically, we tested the effect of the donor genotype on chronic rejection and biliary complications. Our results suggest that donor HLA-C2 may correlate (although not reaching statistical significance) with an increased incidence of chronic rejection (Fig. 3a). No clear effect of donor HLA-C2 was observed on biliary complications (Fig. 3b). Finally, the presence of HLA-C2 in the donor did not influence the incidence of acute rejection (Table 2a).

Discussion

Our results indicate that HLA/KIR mismatches between donor and recipient do not predict LTX outcome. None of the models used here to predict NK-cell alloreactivity has indicated a prominent role of donor or recipient NK cells in graft rejection, graft or patient survival. In addition, our results show that the possession of HLA-C2 by the donor does not influence graft survival, patient survival or acute rejection, confirming what was reported by Tran *et al.* [27].

Donor-versus-recipient NK-cell alloreactivity has been demonstrated for the first time in HSCT [3,12–14]. In this context, the authors have shown that donor NK-cell alloreactivity could prevent leukaemia relapse and graft rejection and protect patients against graft-versus-host disease [12]. The existence of a donor-versus-recipient alloreactivity was also occasionally observed in the context of LTX, since cases of graft-versus-host disease have been recorded [15,16]. However, the specific cell types contributing to the onset of this alloreaction have not been clarified. A previous publication from our group has introduced the hypothesis that, similar to HSCT, a tolerogenic effect due to donor NK-cell alloreactivity may also apply to LTX [17]. This concept was supported by the observation that highly cytotoxic graft-derived NK cells are transferred into the recipient upon LTX [17]. Seeing the universally recognized tolerogenic effects of liver grafts we hypothesized that, similar to HSCT, the large number of donor NK cells transferred into the recipient may be an important factor contributing to graft acceptance by specifically targeting the recipient antigen presenting cells and alloreactive T cells. Herein, we report that the classical models used to predict NK-cell alloreactivity did not indicate a prominent role for donor NK cells in the outcome of LTX.

The role of donor and recipient HLA/KIR interactions in recipient-versus-donor alloreactivity is poorly defined in solid organ transplantation due to conflicting data

from clinical and *in vitro* studies as well as from animal models [18,36–39]. In the context of LTX, conflicting data have been reported. Two recent publications [24,27] reported largely contradicting results on the effects of HLA-C on LTX outcome: while Hanvesakul *et al.* [24] showed improved graft and patient survival when the donor possessed at least one HLA-C2 allele, Tran *et al.* [27] found that the same donor allele had no impact on either graft or patient survival. In addition, contradicting results were published with regard to the effect of NK-cell alloreactivity on liver graft rejection [23–26]. Herein, we substantially add to this discussion by showing that, in our cohort, the possession of HLA-C2 by the donor does not independently affect graft survival, patient survival or acute rejection, confirming what was reported by Tran *et al.*

A few differences among the three studies can be enumerated for a better comparison of the outcomes. As the group of Hanvesakul [24], we also considered patients from one single centre, while the cohort analysed by Tran [27] was multicentre. An additional difference refers to patients' ethnicity: the cohort analysed by Tran included only Caucasians patients, whereas the original study from Hanvesakul was unclear on this aspect, but most probably analysed a multiethnic cohort. Our cohort was composed of multiple ethnicities (Table 1a) and restriction of our study group to Caucasian patients did not change the results as concerning the effect of donor HLA-C2 on LTX outcome (Fig. 2). Finally, while the study from Hanvesakul excluded patients with graft survival of <30 days, Tran included all patients available, both primary LTX and re-LTX procedures (which represented 6.4% of the whole cohort). For our study we included only patients with primary LTX and graft survival of >7 days, since graft loss in the first week is, in general, mostly due to surgical complications and less related to immunological factors. The decision of excluding re-LTX cases from our cohort was based on the evidence that re-transplanted patients, having a significantly worse survival than primary transplants [40,41], may add a confounding effect to the outcome of the analysis.

Further analyses were performed to estimate the association of NK-cell alloreactivity, as predicted by accepted models such as 'missing self' or 'missing ligand', with LTX outcome. While 'missing self' considering both HLA-C and -Bw4 (or each single epitope considered alone) did not significantly associate with LTX outcome, we initially observed a univariate association with better graft survival when the donor missed the HLA-Bw4 allele present in the recipient's genotype. This trend, however, was completely abrogated in multivariate analysis. Our observation that missing-self considering HLA-C alone is not associated with acute rejection after LTX confirms similar results from three other groups [24–26].

Several levels of complication are intrinsically characterizing NK-cell biology and are still not completely elucidated: the licensing process of NK-cell education [42–44]; the dynamics determining that a certain KIR present at the DNA level is effectively expressed and functional at the cell surface [45–47]; the kinetics determining the stronger/weaker affinity of each inhibitory KIR compared to its activating counterpart [48]; the hierarchy determining the strength of inhibition of different HLA alleles [32,49–51]. In addition, the attempt to investigate the effects of NK-cell alloreactivity on LTX outcome is further complicated by the variation of a number of clinical parameters that differ among patients, such as the underlying disease and the immunosuppressive treatment. Different underlying diseases may affect *a priori* the functionality of recipient NK cells and influence the basal functional competence of NK cells [52–54]. On the other hand, post-LTX immunosuppressive treatment may have an effect on NK cell function. In this sense, although immunosuppressive drugs appear to have a modest effect on NK cells [6,19], few studies have reported an altered function of NK cells as a consequence of various immuno suppressants [55]. Finally, interpreting correlations between HLA genotype and transplantation outcome is complicated by the existence of additional factors, such as viral infections, that *in vivo* can mask the direct effects of the sole genotype. Increasing evidence is indeed showing how these factors can either generate alloreactivity even when not predicted by HLA disparities [56,57], or can promote immune regulation even in an unfavourable HLA mismatched environment [58,59]. However, since current experimental techniques do not allow direct quantification of alloreactivity of bulk NK cells [12,13], models that predict NK-cell alloreactivity on basis of KIR and HLA genotypes are presently the best approach available.

Overall, based on the current models of NK-cell alloreactivity, we could not find an association between predicted NK-cell alloreactivity and LTX outcome in both donor-versus-recipient and recipient-versus-donor direction. We support the concept that our current understanding of the biological mechanisms underlying NK-cell alloreactivity is still not complete and does not allow for the definite identification of their role, if any, in solid organ transplantation and LTX in particular. Ultimately, as already proposed by Tran and the editorial accompanying their article [60], this field remains quite 'unresolved' and only further and more clinical/biological-integrated research will possibly lead to more clear indications.

Authorship

VM: contributed to the creation of the database, performed the data analysis and participated in the writing

of the article. AvdM and IJ: participated in study design, supervised HLA-C and KIR typing, participated in the writing of the article and contributed to the discussion of the results. HWT, GK and LJWvdL: contributed in the writing of the article. HJM: performed the evaluation of clinical cases, participated in study design and contributed in the writing of the article. JK: participated in study design, in the writing of the article, contributed to the discussion of the results and supervised the study.

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