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Peptide Sharing Between CMV and Mismatched HLA Class I Peptides Promotes Early T-Cell-Mediated Rejection After Kidney Transplantation

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ABSTRACT

Cytomegalovirus (CMV) infection is related to acute rejection and graft loss after kidney transplantation, though the underlying mechanism remains largely unknown. Some CMV strains produce a peptide that is identical to a peptide sequence found in the leader peptide of specific HLA-A and -C alleles. In this retrospective study of 351 kidney transplantations, we explored whether CMV-seropositive recipients without the VMAPRTLIL, VMAPRTLLL or VMAPRTLVL HLA class I leader peptide receiving a transplant from a donor with this peptide, faced an increased risk of T-cell-mediated rejection (TCMR) in the first 90 days after transplantation. An independent case–control cohort was used for validation ($n = 122$). The combination of recipient CMV seropositivity with the VMAPRTLIL peptide mismatch was associated with TCMR with a hazard ratio (HR) of 3.06 ($p = 0.001$) in a multivariable analysis. Similarly, the VMAPRTLLL peptide mismatch was associated with TCMR revealing a HR of 2.61 ($p = 0.008$). Transplantations featuring either a VMAPRTLIL or a VMAPRTLLL peptide mismatch had a significantly higher cumulative TCMR incidence ($p < 0.0001$), with the primary impact observed in the first 2 weeks post-transplantation. The findings could be validated in an independent cohort. Together, our data strongly suggest that CMV-positive recipients without an HLA peptide identical to a CMV peptide yet transplanted with a donor who does possess this peptide, have a significantly increased risk of early TCMR. Considering the prevention of such an leader peptide mismatch in these patients or adjusting immunosuppression protocols accordingly may hold promise in reducing the incidence of early TCMR.

1 | Introduction

Cytomegalovirus (CMV) infection is an important cause for mortality after kidney transplantation [1–4], and is associated with rejection and graft loss [4–7]. A systematic review on CMV and transplant rejection [8] showed that kidney transplant recipients with CMV infection or disease were twice as likely to develop acute rejection as compared to recipients without CMV infection or disease. Despite its significant impact, the underlying mechanisms driving the association between CMV and graft rejection remain poorly understood. Potentially, reduction of immunosuppressive therapy at time of the diagnosis of CMV

disease, CMV-induced upregulation of cytokines, adhesion molecules and HLA class II molecules [9] or other immune-mediated mechanisms may be involved.

Given that CMV infection in the recipient significantly affects T-cell-mediated rejection, a role for T-cell epitopes can be suspected [10–12]. Epitope sharing or molecular mimicry between CMV epitopes and mismatched HLA epitopes may underlie the immune-mediated mechanisms contributing to the intricate relationship between CMV and kidney graft rejection. More specifically, in instances where a recipient has been previously immunised against a CMV-derived epitope shared with a donor HLA epitope, a rapid and heightened immune response could be triggered upon transplantation due to the presence of T-cell memory against the shared epitope. Similar to sharing of T-cell epitopes between earlier transplants, this heightened response against the mismatched donor HLA-derived peptide may enhance graft failure, further complicating the post-transplantation outcome [13].

The phenomenon of peptide sharing has been described in the context of CMV. In specific CMV strains, the UL40 glycoprotein contains a nonameric peptide sequence that can bind to the non-classical HLA-E, and is also present in the leader peptide of specific HLA class I alleles [14]. Recipients who possess at least one HLA allele containing the specified peptide are expected to be tolerant to this peptide. As a result, they should not mount an immune response against the CMV peptide that shares the same amino acid sequence. Conversely, patients without this peptide in their HLA alleles may develop an immune response against this CMV peptide. The presence of T cells directed against CMV UL40-derived peptides restricted by HLA-E have been reported in multiple studies [14–16], with one study reporting that around a quarter of CMV-seropositive individuals possess an HLA-E/UL40 CD8-positive T-cell response [16].

The presence of such HLA-E-restricted UL40-specific cells may have consequences in the context of transplantation, as these T cells can recognise allogeneic cells [14, 17]. Kidney transplant recipients who previously developed an immune response against the UL40 peptide, may react to donor cells expressing the HLA class I peptide identical to the UL40 peptide. This recognition could subsequently increase the risk of early T-cell-mediated rejection. Indeed, in a small cohort of 15 lung transplant recipients possessing an HLA type allowing for the generation of UL40-specific T cells, HLA-E-restricted UL40-specific T cells were detected in seven patients. Six of these patients experienced acute cellular rejection [18], suggesting that these cells could indeed play a significant role during acute cellular rejection. Yet, large clinical studies confirming the negative impact of T-cell epitope sharing between CMV and mismatched HLA are lacking.

In the present retrospective study, we aimed to elucidate the association between CMV and rejection after kidney transplantation by addressing this peptide mismatch. Our findings indicate that a positive CMV serostatus of a transplant recipient combined with a mismatch of the UL40-identical VMAPRTLIL or VMAPRTLLL peptide strongly increases the risk of developing early TCMR after kidney transplantation.

2 | Methods

2.1 | Study Populations

Patients with end-stage kidney failure who underwent a kidney transplantation at the Utrecht Medical Center between 2006 and 2015 were eligible for inclusion in the present retrospective study. The study included patients who received a kidney transplant from either a living or a deceased donor. All patients had a pre-transplant negative T- and B-cell complement-dependent cytotoxicity assay crossmatch. Clinical outcomes were derived from the Dutch Organ Transplantation Registry (NOTR). Patients had supplied informed consent for this registry. Repeat transplantations ($n = 110$) were excluded to minimise the number of pre-immunised recipients in the cohort. After additional exclusion of transplantations where the CMV serostatus of the recipient was unknown ($n = 20$), transplantations with incomplete donor and/or recipient HLA-A, -B, -C and -DR typing ($n = 226$), and patients with primary non-function ($n = 7$), a total of 351 patients were included in this study. HLA typing was performed by PCR using sequence-specific oligonucleotides (PCR-SSO, OneLambda), delivering an HLA typing at intermediate resolution for all included kidney transplant donors and recipients for a minimum of HLA-A, -B, -C and -DR. The resulting typing ambiguities resulted in an unambiguous assignment of the leader peptide sequence for all included cases. As an independent validation cohort, 122 kidney transplantations that were performed at the Hospital do Rim, UNIFESP, São Paulo, Brazil, were analysed. These transplantations were part of a retrospective single-centre case–control study, where the case group comprised recipients with treated rejection within the first 3 months after transplantation, and the control group recipients that received a kidney from the same donor but did not present with rejection. All patients had a pre-transplant negative T- and B-cell complement-dependent cytotoxicity crossmatch without HLA-A, -B and -DR donor-specific antibodies (MFI $\geq$ 1,500). All donors and recipients were typed for HLA at second-field high-resolution level for HLA-A, -B, -C, -DRB1 and -DQB1. The study was approved by the local ethics committee (Federal University of São Paulo, approval number 27785119.2.0000.5505). This study was conducted in accordance with the Declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'.

2.2 | Study Covariates

For the hypothesis-generating cohort, relevant clinical data were extracted from the University Medical Center Utrecht databases and the NOTR. Recipient-related covariates encompassed age, sex, percentage panel reactive antibodies (PRA, determined using complement-dependent cytotoxicity crossmatch), number of previous pregnancies and CMV serostatus. Donor-related covariates included age, sex, the type of donor (i.e., living or deceased donor) and CMV serostatus. Transplant-related covariates included the year of transplantation. For the validation cohort, the covariates of interest were obtained from

clinical files. PIRCHE-II scores, as an indicator for the number of donor-derived T-cell epitopes [19], were determined using the PIRCHE-II algorithm (version 3.3.69, available via pirche.com). PIRCHE-II scores were logarithmically transformed [20].

2.3 | Clinical Outcomes

The primary end point of this study was TCMR within the first 90 days after transplantation. Based on the cumulative TCMR incidence graphs, TCMR within the first 14 days after transplantation was used as outcome measure for subsequent analyses. Patients with deterioration in kidney function and/or unexplained proteinuria underwent an indication biopsy to validate suspicion of rejection of the transplanted kidney. In the validation cohort, acute rejections were either clinically diagnosed, based on response to treatment ($n = 8$), or biopsy-proven ($n = 53$). For both cohorts, biopsy-proven instances of TCMR were classified according to the Banff classification [21].

2.4 | Peptide Subgroups

To determine the group of patients at risk, we identified the transplantations where the recipient was seropositive for CMV, where the HLA peptide identical to a CMV peptide was not present in the HLA typing of the recipient, and where this same peptide was present in the HLA typing of the donor. To this extent, first, the different HLA class I alleles were classified according to their peptide. Three variations to the same UL40-peptide that are produced by CMV also occur in the leader peptide of specific HLA class I alleles: VMAPRTLIL (the 'LIL' peptide) present in specific HLA-C alleles, VMAPRTLLL (the 'LLL' peptide) present in specific HLA-A and HLA-C alleles and VMAPRTLVL (the 'LVL' peptide) present in specific HLA-A alleles (Table 1). Leader peptide sequences were obtained via IPD-IMGT/HLA (release 3.54 (2023–10)) [22]. Next, the transplantations were divided into subgroups for the LIL peptide, the LLL peptide and the LVL peptide.

2.5 | Statistical Analyses

Statistical analyses were performed using R version 4.3.0. Baseline characteristics between groups were compared using a Mann–Whitney *U*-test for continuous variables and a chi-squared or a Fisher's Exact test for categorical variables. Normality was tested using a Shapiro–Wilk test. To identify the risk factors for TCMR in the first 90 days after transplantation, the combination of recipient CMV seropositivity and a VMAPRTLIL, VMAPRTLLL or VMAPRTLVL peptide mismatch, as well as other factors which are known to influence transplantation outcome, were studied in a Cox proportional hazard model. The proportional hazards assumption was assessed for all variables using the Schoenfeld residuals against the transformed time. In case of violation of the assumption, a time-dependent covariate was constructed using the time-transforming functionality within the survival package in R. The covariates found to be associated with the outcome with a p-value of 0.1 or lower were subsequently entered into a multivariable Cox proportional hazard model. Patients were censored at their last confirmed visit. Cumulative TCMR incidence was analysed using a Kaplan Meier analysis. A p-value < 0.05 was considered statistically significant.

3 | Results

3.1 | Baseline Characteristics

A total of 351 transplant pairs were eligible for inclusion in the hypothesis-generating cohort. Donor and recipient baseline characteristics are summarised in Table 2. TCMR was observed in 88 (25.1%) patients, of which 65 experienced TCMR within 90 days after transplantation. Throughout follow-up, 49 patients (14.0%) had graft failure. The validation cohort contained 122 transplantations performed in Brazil that were part of a case–control study, where two recipients received a kidney from the same donor and where one of the two developed TCMR within 90 days after transplantation ($n = 61$), and

TABLE 1 | Peptides that occur in the UL40 protein of specific CMV strains as well as in the leader peptide of HLA class I alleles.

Peptide	Example of a CMV strain containing the peptide	Approximate frequency of strains containing the peptide[a]	HLA class I alleles
VMAPRTLIL	AD169 (UniProt P16780)	~36%	HLA-C*01, -C*03, -C*04, -C*05, -C*06, -C*08:01–03, -C*12, -C*14, -C*16 and -C*17:02
VMAPRTLLL	Towne (UniProt D5LX51)	~21%	HLA-A*01, -A*03, -A*11, -A*29, -A*30, -A*31, -A*32, -A*33, -A*36 and -A*74 HLA-C*02 and -C*15
VMAPRTLVL	Toledo (UniProt D3YRX8)	~11%	HLA-A*02, -A*23, -A*24, -A*25, -A*26, -A*34:02, -A*43, -A*66 and -A*69

Abbreviation: CMV: cytomegalovirus.
[a]Frequencies are based on frequencies reported in Vietzen et al. [23] and Hammer et al. [24]. Peptides produced by CMV that are not shared with the leader peptides of classical HLA class I alleles have not been not included in this table.

TABLE 2 | Baseline characteristics of the Dutch hypothesis-generating cohort ($n = 351$) and Brazilian validation cohort ($n = 122$).

		Validation cohort (Brazil, $n = 122$)		
	Hypothesis-generating cohort (The Netherlands, $n = 351$)	**Acute rejection ($n = 61$)**	**No acute rejection ($n = 61$)**	***p value*[h]**
Recipient characteristics				
Age recipient (median, IQR)	55 (43–64)	42 (35–53)	41 (33–55)	0.77
Sex recipient (male, %)	217 (61.8%)	46 (75.4%)	36 (59.0%)	0.08
CMV serostatus recipient (positive, %)	197 (56.1%)	57 (93.4%)	55 (90.2%)	0.74
Previous pregnancies (yes, % of all women)[a]	80 (66.1%)	12 (80.0%)	14 (56.0%)	0.23
Panel reactive antibodies (PRA) (*n*, %)[b]				0.93
< 5%	320 (91.2%)	48 (78.7%)	49 (80.3%)	
5%–85%	27 (7.7%)	9 (14.8%)	9 (14.8%)	
> 85%	4 (1.1%)	4 (6.6%)	3 (4.9%)	
Donor characteristics				
Age donor (median, IQR)	55 (47–62)	47 (33–55)	47 (33–55)	—
Sex donor (male, %)	157 (44.7%)	34 (55.7%)	34 (55.7%)	—
CMV serostatus donor (positive, %)[c]	90 (50.3%)	54 (91.5%)	54 (91.5%)	—
Type donor (living, %)	171 (48.7%)	N.A.	N.A.	—
Transplantation characteristics				
Sex mismatch (*n*, %)				0.17
Matched	161 (45.9%)	35 (57.4%)	29 (47.5%)	
Female recipient, male donor	65 (18.5%)	7 (11.5%)	15 (24.6%)	
Male recipient, female donor	125 (35.6%)	19 (31.1%)	17 (27.9%)	
Transplantation year (median, IQR)	2012 (2010–2014)	2016 (2015–2017)	2016 (2015–2017)	—
PIRCHE-II score (median, IQR)[d]	59 (33–86)	50 (28–71)	57 (36–85)	0.30
Graft failure/loss (*n*, %)[e]	49 (14.0%)	11 (18.0%)	4 (6.6%)	0.10
TCMR (*n*, %)	88 (25.1%)	61 (100%)[g]	N.A.	—
Borderline	22 (25.0%)	21 (34.4%)		
Banff Grade 1 (tubulo-interstitial rejection)	25 (28.4%)	15 (24.6%)		
Banff Grade 2 (vascular rejection)	40 (45.5%)	16 (26.2%)		
Banff Grade 3	1 (1.1%)	1 (1.6%)		
Unknown[f]	N.A.	8 (13.1%)		
TCMR within 90 days post-Tx (*n*, %)	65 (18.5%)	61 (100%)[g]	N.A.	—
Borderline	14 (21.5%)	21 (34.4%)		
Banff Grade 1 (tubulo-interstitial rejection)	16 (24.6%)	15 (24.6%)		
Banff Grade 2 (vascular rejection)	34 (52.3%)	16 (26.2%)		

(Continues)

TABLE 2 | (Continued)

	Hypothesis-generating cohort (The Netherlands, $n = 351$)	Validation cohort (Brazil, $n = 122$)		
		Acute rejection ($n = 61$)	No acute rejection ($n = 61$)	*p value*[h]
Banff Grade 3	1 (1.5%)	1 (1.6%)		
Unknown[f]	N.A.	8 (13.1%)		

Abbreviations: CMV: cytomegalovirus; IQR: interquartile range; PIRCHE: Predicted Indirectly ReCognisable HLA Epitopes; TCMR: T-cell-mediated rejection.
[a]13 missing in hypothesis-generating cohort.
[b]In the hypothesis-generating cohort, the PRA was determined using complement-dependent cytotoxicity assay (CDC). In the validation cohort, the PRA is based on unacceptable antigens (vPRA).
[c]172 missing in hypothesis-generating cohort, 2 missing in validation cohort (4 transplantations).
[d]2 missing in hypothesis-generating cohort.
[e]Graft failure in hypothesis-generating cohort, graft loss in validation cohort.
[f]Clinically diagnosed (based on response to treatment).
[g]The number of patients experiencing TCMR and TCMR within 90 days after transplantation are identical for the validation cohort, as this cohort only includes transplantations where the recipient who developed TCMR, experienced the TCMR episode within 90 days after the transplantation.
[h]For comparing baseline characteristics between recipients with and without acute rejection, *p* values were calculated using a Wilcoxon signed rank test for the numerical variables. For categorical variables, a chi-squared test was used.

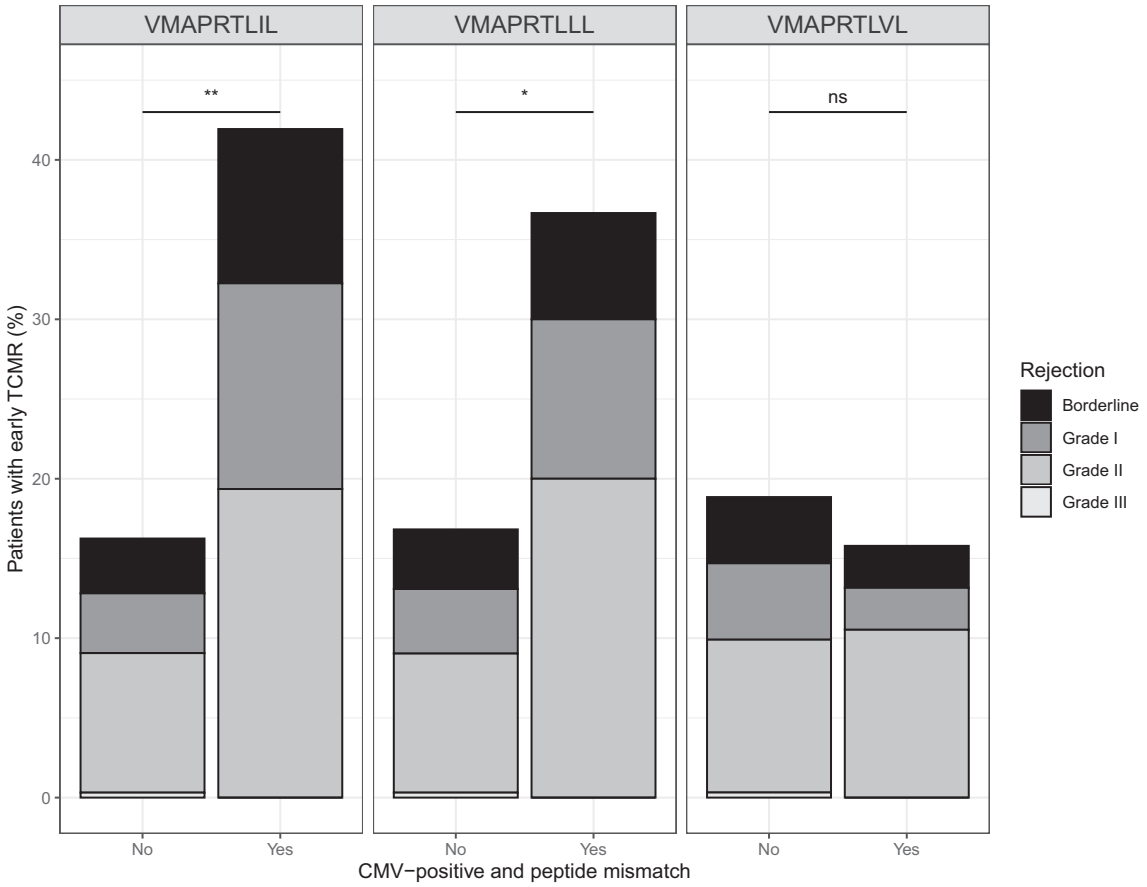

FIGURE 1 | Percentage of recipients experiencing early TCMR (< 90 days post-Tx) among recipients with and without CMV seropositivity and a peptide mismatch in the Dutch cohort ($n = 351$), separated per peptide. Left panel (LIL peptide): 52/320 (16.3%) TCMR in the group without CMV and/or LIL peptide mismatch, 13/31 (41.9%) TCMR in the CMV-seropositive group with the LIL peptide mismatch. Middle panel (LLL peptide): 54/321 (16.8%) TCMR in the group without CMV and/or LLL peptide mismatch, 11/37 (36.7%) TCMR in the CMV-seropositive group with LLL peptide mismatch. Right panel (LVL peptide): 59/313 (18.8%) TCMR in the group without CMV and/or LVL peptide mismatch, 6/38 (15.8%) TCMR in the CMV-seropositive group with the LVL peptide mismatch. CMV: cytomegalovirus; TCMR: T-cell-mediated rejection.

TABLE 3 | Factors associated with T-cell-mediated rejection in the Dutch cohort ($n = 351$).

	Univariable		Multivariable	
	HR (95% CI)	***p***	**HR (95% CI)**	***p***
Age recipient, per 10 years increment[a]	0.98 (0.94–1.02)	0.30		
Sex recipient (*ref: female*)	0.89 (0.54–1.45)	0.63		
CMV serostatus recipient	**1.59 (0.95–2.66)**	**0.08**	0.94 (0.52–1.72)	0.85
Previous pregnancies	0.72 (0.31–1.68)	0.45		
PRA yes/no	**2.65 (1.49–4.71)**	**0.001**	**2.45 (1.37–4.39)**	**0.003**
Age donor, per 10 years increment	1.14 (0.91–1.43)	0.24		
Sex donor (*ref: female*)[a]	1.00 (0.86–1.16)	1.00		
CMV serostatus donor	0.96 (0.49–1.88)	0.91		
Type donor (*ref: deceased*)	0.94 (0.58–1.53)	0.80		
Transplantation year	1.07 (0.97–1.19)	0.18		
ln (PIRCHE-II + 1)	**1.67 (1.18–2.36)**	**0.004**	**1.58 (1.10–2.26)**	**0.01**
Recipient CMV seropositive and VMAPRTLIL mismatch (*ref: recipient seronegative and/or no VMAPRTLIL mismatch*)	**2.97 (1.62–5.45)**	**< 0.001**	**3.06 (1.55–6.02)**	**0.001**
Recipient CMV seropositive and VMAPRTLLL mismatch (*ref: recipient seronegative and/or no VMAPRTLLL mismatch*)	**2.61 (1.37–5.00)**	**0.004**	**2.61 (1.28–5.33)**	**0.008**
Recipient CMV seropositive and VMAPRTLVL mismatch (*ref: recipient seronegative and/or no VMAPRTLVL mismatch*)	0.80 (0.35–1.86)	0.61		

Note: In the multivariable model, all variables that had a p value < 0.1 in the univariable model were included. Values in bold indicate variables with a p value < 0.05.
Abbreviations: CMV: cytomegalovirus; PIRCHE: Predicted Indirectly ReCognisable HLA Epitopes; PRA: panel reactive antibodies.
[a]For these variables, the proportional hazards assumption was violated. Consequently, a time-dependent covariate was constructed from these variables.

the other did not ($n = 61$). In this cohort, 91.8% of the patients were CMV-seropositive. None of the variables differed significantly between cases with and without rejection (Table 2).

3.2 | VMAPRTLIL Peptide Mismatching Enhances Early TCMR in CMV-Seropositive Recipients

We subsequently assessed whether peptide sharing between CMV and HLA Class I could impact TCMR in the first 90 days after transplantation. To this end, transplantations with CMV-seropositive recipients without VMAPRTLIL (the 'LIL' peptide), VMAPRTLLL (the 'LLL' peptide) or VMAPRTLVL (the 'LVL' peptide) in their HLA class I alleles were separated into three groups; those receiving a kidney from a donor expressing HLA alleles containing the LIL, LLL or LVL peptide, respectively (Table 1).

CMV-seropositive recipients with the LIL peptide mismatch experienced significantly more frequent TCMR compared to transplantations without recipient CMV seropositivity and/or the LIL peptide mismatch (41.9% and 16.3%, respectively, $p = 0.001$) (Figure 1). For the LLL peptide, a similar effect was observed (36.7% and 16.8%, respectively, $p = 0.02$). In contrast, no significant difference in the occurrence of TCMR between transplantations with and without the LVL peptide mismatch was found (15.8% and 18.8, respectively, $p = 0.81$). None of the subgroups showed differences in the severity of the rejection episodes, as classified using Banff grades (Figure 1).

The LIL, LLL and LVL peptide-mismatched transplantations with and without recipient CMV seropositivity did not differ significantly for the majority of the baseline characteristics analysed (Tables S1–S3). However, for the LLL peptide, the PIRCHE-II scores were significantly higher among the transplantations with the peptide mismatch compared to the transplantation without the mismatch ($p = 0.01$). A similar trend was observed for the LIL and the LVL peptide. To assess whether the observed difference in PIRCHE-II scores between the two groups is the consequence of the mismatched peptide only, or due to the mismatched peptide combined with a positive CMV serostatus, the PIRCHE-II scores of the LIL, LLL

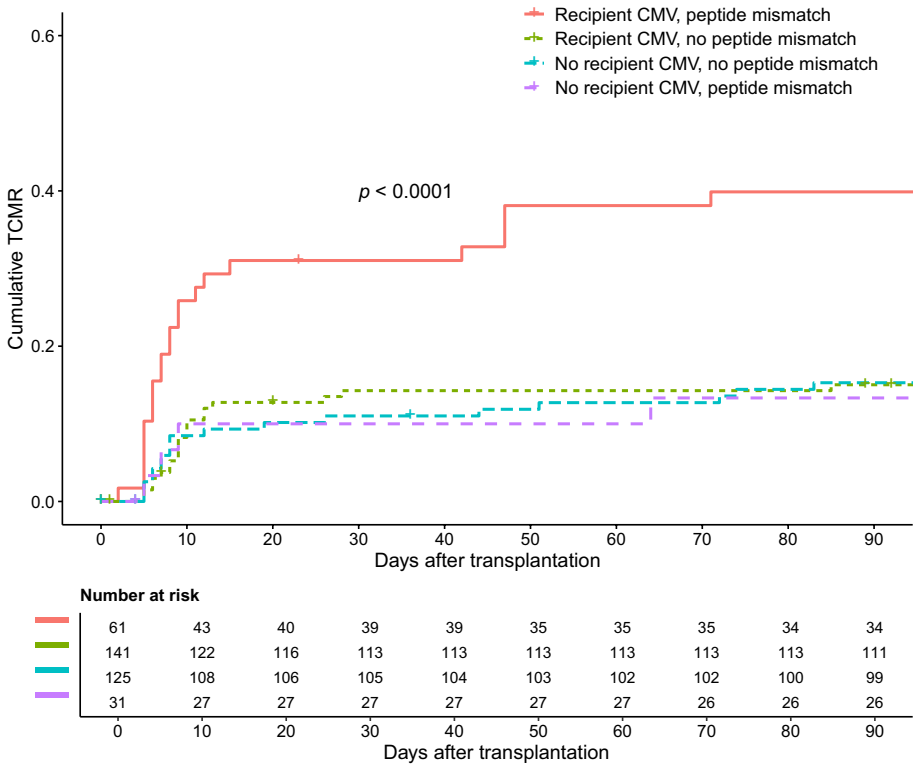

FIGURE 2 | Cumulative TCMR for LIL- and LVL-peptide mismatched subgroups combined in the Dutch cohort ($n = 351$), up to 90 days after transplantation. The red line indicates transplantations with a CMV-seropositive recipients and a LIL or LLL peptide mismatch. The p value depicted is the p value of the red line compared to the other three groups combined. CMV: cytomegalovirus; TCMR: T-cell-mediated rejection.

and LVL peptide-mismatched transplantations with a seropositive recipient were compared with the PIRCHE-II scores of the peptide-mismatched transplantations with a seronegative recipient. The PIRCHE-II scores did not differ between the two groups ($p = 0.92$, $p = 0.80$ and $p = 0.27$, respectively).

CMV-seropositive recipients without the LIL peptide transplanted with a donor with the LIL peptide (e.g., HLA-C*01, -C*03 or -C*06) had a significantly increased risk of developing early TCMR with a HR of 2.97 (95% CI 1.62–5.45, $p < 0.001$, univariable Cox proportional hazards analysis) (Table 3). This effect was only observed for the interaction between CMV serostatus and LIL peptide mismatching; the HR of a mismatch of the LIL peptide only, independent of CMV serostatus of the recipient, was lower than the HR of the peptide mismatch combined with the CMV serostatus of the recipient (HR 1.87, 95% CI 1.05–3.33, $p = 0.03$). A similar effect was observed for the LLL peptide, with a HR of 2.61 (95% CI 1.37–5.00, $p = 0.004$). In contrast, a positive CMV serostatus of the recipient combined with a LVL mismatch did not result in an increased risk for developing early TCMR (HR 0.80, 95% CI 0.35–1.86, $p = 0.61$).

After correction for potentially confounding variables as identified in the univariable Cox analysis, both the LIL and the LLL peptide mismatch remained associated with early TCMR (Table 3). The LIL peptide mismatch was associated with early TCMR with a HR of 3.06 (95% CI 1.55–6.02, $p = 0.001$), and the LLL peptide mismatch was associated with early TCMR with a HR of 2.61 (95% CI 1.28–5.33, $p = 0.008$).

Recipients who were CMV seropositive and had either the LIL or the LLL peptide mismatch had a significantly higher cumulative TCMR in the first 90 days after transplantation compared to the patients who did not have this combination (Figure 2). This effect was mainly observed in the first 2 weeks after transplantation. After stratification for the LIL and LLL peptide, the effect remained prominent for both peptides (Figure 3, $p < 0.001$ and $p < 0.01$, respectively). Notably, among the transplantations without the combination of a CMV-seropositive recipient and a LIL or LLL peptide mismatch, no differences were found between CMV-seropositive and -seronegative patients (Figure 2; HR 1.01, 95% CI 0.55–1.85, $p = 0.98$), indicating that the association between CMV and TCMR is caused by the LIL and/or LLL peptide mismatch.

3.3 | Effect of VMAPRTLIL and VMAPRTLLL Peptide Mismatching Is Confirmed in an Independent Cohort

To confirm our findings, we analysed the presence of the LIL, LLL and LVL peptide mismatch in an independent case–control cohort consisting of 122 Brazilian kidney transplantations. The effects for the LIL and LLL peptide mismatch could be confirmed: recipients experiencing TCMR had significantly more often a LIL mismatch or an LLL mismatch (Figure 4, $p = 0.02$ and $p = 0.03$, respectively). More specifically, CMV-seropositive recipients with a LIL mismatch have odds of developing TCMR in the first 3 months after the transplantation that are 3.94 times those that do not have this mismatch (95%

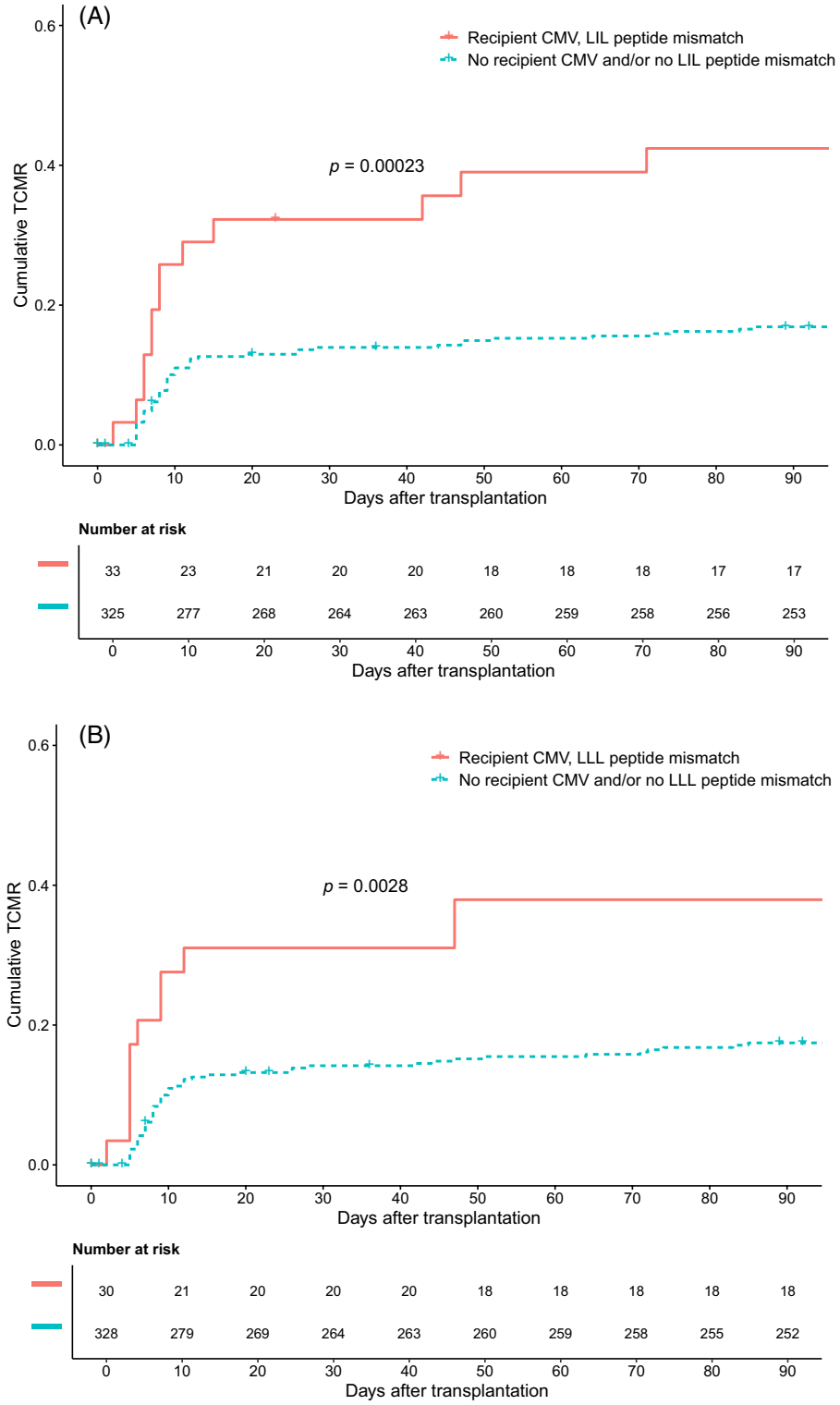

FIGURE 3 | Cumulative TCMR in the Dutch cohort ($n = 351$) up to 90 days after transplantation, divided by LIL peptide mismatch and LLL peptide mismatch. (A) Cohort divided by CMV-seropositivity and LIL peptide mismatch. The red line indicates transplantations with a CMV-seropositive recipients and a LIL peptide mismatch. (B) Cohort divided by CMV-seropositivity and LLL peptide mismatch. The red line indicates transplantations with a CMV-seropositive recipients and a LLL peptide mismatch. CMV: cytomegalovirus; TCMR: T-cell-mediated rejection.

CI 1.26–14.83, $p = 0.02$). For the LLL peptide mismatch, the odds ratio was 5.71 (95% CI 1.14–55.94, $p = 0.03$). Paralleling the findings of the main cohort, no effect for the LVL peptide mismatch was found (Figure 4 right panel, OR 0.89, 95% CI 0.27–2.77, $p = 1.00$).

3.4 | Differential Proteasomal Cleavage May Affect Risk of TCMR

Although the peptide flanking regions of all HLA class I alleles containing the LIL or LVL leader peptide are

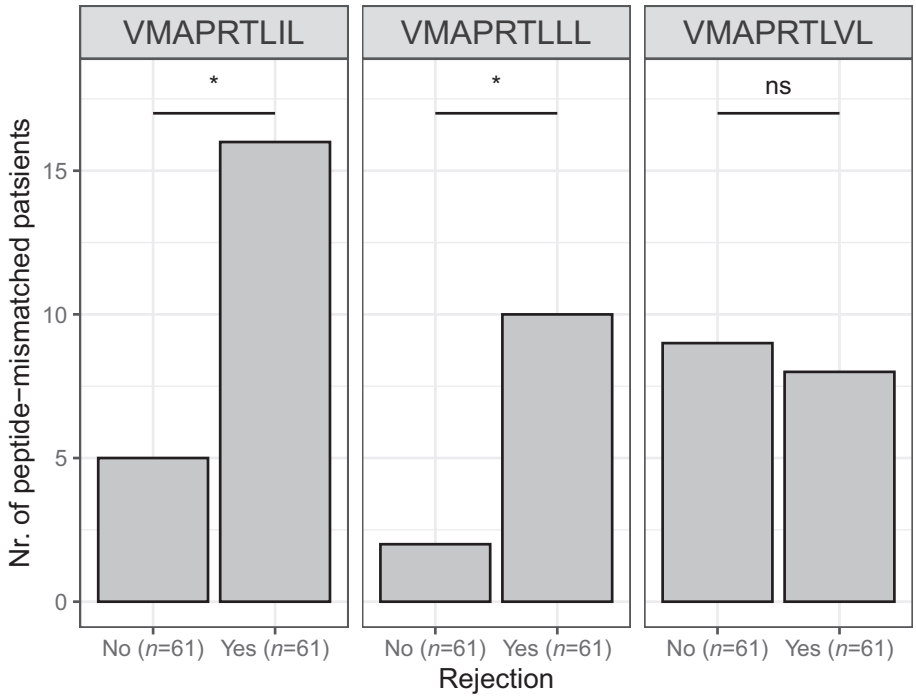

FIGURE 4 | Number of CMV-seropositive peptide-mismatched recipients (VMAPRTLIL left, VMAPRTLLL middle and VMAPRTLVL right panel) in the case group (recipients developing rejection within 3 months, $n = 61$) and the control group (recipients without rejection in the first 3 months after transplantation, $n = 61$) in the Brazilian validation cohort ($n = 122$).

TABLE 4 | Flanking amino acid sequences of the VMAPRTLLL leader peptide.

Peptide sequence	HLA Class I alleles
MR | VMAPRTLLL | ***LLS***GALAL ...	HLA-C*02, -C*15
MA | VMAPRTLLL | ***LLS***GALAL ...	HLA-A*01, −A*03, −A*11, −A*30, −A*36
MA | VMAPRTLLL | ***LLL***GALAL ...	HLA-A*29, −A*31, −A*32, −A*33, −A*74

identical (**MR**VMAPRTLIL**LLS** and **MA**VMAPRTLVL**LLS**, respectively), LLL leader peptides show differences depending on the specific HLA-C allele (Table 4). Such flanking differences could potentially affect proteasomal-mediated cleavage. More specifically, while an arginine (R) or an alanine (A) at P2 does not seem to influence HLA-E expression, the presence of VMAPRTLLL***LLS***, but not VMAPRTLLL***LLL***, has been shown to decrease HLA-E cell surface expression [25]. Hypothetically, VMAPRTLLL***LLS*** peptide mismatches, but not VMAPRTLLL***LLL*** mismatches, increase the risk for developing early TCMR. Separating LLS from LLL peptides yield a HR of 2.89 (95% CI 1.40–5.98, $p = 0.004$) to 3.33 (95% 1.76–6.30, $p < 0.001$). In contrast, the increased risk for early TCMR seemed to disappear when considering the VMAPRTLLL***LLL*** peptide mismatch only (HR 1.42, 95% CI 0.56–3.59, $p = 0.46$). A potential effect for the VMAPRTLLL***LLS*** peptide mismatch specifically could not be validated in the Brazilian cohort (Odds ratio 0.87, 95% CI 0.27–2.77, $p = 1.00$).

4 | Discussion

Associations between CMV and rejection after transplantation have been reported before [4–7]. Yet, the underlying mechanism remains largely unknown. Here, we retrospectively investigated whether CMV-seropositive patients without an HLA class I leader peptide identical to a UL40 peptide produced by certain CMV strains and transplanted with a donor with this peptide, have an increased risk of early TCMR. We here for the first time demonstrated a strong and significant interaction between a CMV UL40 peptide and mismatched HLA class I leader peptides in relation to early TCMR. CMV-seropositive recipients with an LIL or LLL peptide mismatch had over a 2- and 3-fold increased risk of developing TCMR within 90 days after transplantation, respectively, compared to recipients who are CMV-seronegative or do not have an HLA class I leader peptide mismatch. This effect was not solely due to CMV serostatus, as the association between CMV and early TCMR was less prominent and not significant (Table 3). The effect observed in this study occurred almost exclusively in the first 2 weeks, suggesting that the generated response likely relates to memory responses previously generated upon CMV infection, eliciting a swift and potent immune response after reactivation by the donor kidney [26]. Indeed, $CD8^+$ T-cell responses towards UL40 and restricted by HLA-E have been found to represent a large and long-lasting pool of T cells [16].

The association between the LIL and LLL peptide mismatch could be confirmed in an independent cohort. For the LVL peptide, both the Dutch and the Brazilian cohort showed no association between the peptide mismatch and TCMR, even though LVL-specific T cells have been reported before [14]. Potentially,

this lack of effect for the LVL peptide could be due to a lower incidence of LVL peptide CMV strain compared to LIL and LLL peptide strains: whereas for the LIL and LLL peptide, around 20–40% of the CMV strains in a European Caucasian population contains this peptide, this percentage is notably lower for the LVL peptide [23, 24]. Additionally, the LVL peptide has been shown to induce effector functions in NKG2C$^+$ natural killer (NK) cells to a lesser extent as compared to the LIL peptide [24], which could suggest that despite the occurrence of this peptide in a subset of patients, a less potent immune response was generated.

Heterologous immunity may provide an alternative explanation for the association between CMV and rejection [27]. Pathogen-specific T cells can be cross-reactive against mismatched donor HLA via direct recognition [27]. In transplantation, the presence of cross-reactive CMV-specific T cells that directly recognise intact mismatched HLA have been reported in multiple individuals [28–32]. For instance, cross-reactivity of an HLA-A1-restricted CMV-pp50-specific T-cell clone against cell lines expressing mismatched HLA-A11 has been reported [28]. To address the relevance of this concept, we analysed the relative presence of these previously reported HLA combinations in the TCMR positive and negative groups with and without CMV in the hypothesis-generating cohort. For instance, the occurrence of TCMR within the combinations of HLA-A1-positive recipient and an HLA-A11-positive donor was compared to the occurrence of TCMR in transplantations without this combination. No evidence for a clinical impact of cross-reactive T cells was found in our cohort (data not shown). Yet, our findings do not exclude an effect in individual patients, as the number of transplantations with a specific HLA combination were low. In addition, heterologous immunity may be a broad phenomenon, which makes modelling hard as it requires knowledge of all cross reactivities. A potential additional role for heterologous immunity should therefore be addressed in bigger cohorts.

Approximately 35% of all recipients at risk according to CMV serostatus and HLA peptide mismatch developed TCMR in the hypothesis-generating cohort. Focusing on the recipients who did not develop TCMR, not all transplantation with an LLL peptide mismatch may have had an LLL-specific immune response, as the flanking amino acids of this peptide seemed to affect the risk of TCMR in the hypothesis-generating cohort, potentially because not all peptide variants can be processed by the proteasome [25]. However, as these findings could not be confirmed in the validation cohort, likely due to limited numbers, validation in a bigger cohort is needed. In addition, some of the patients at risk could also have experienced subclinical rejection, as surveillance biopsies were not performed in this cohort. Finally, the presence of a memory immune response against the CMV peptides that could be reactive against the donor kidney depends on the peptide present in the CMV strain by which the recipient was infected. For the current cohort, details on the specific UL40 peptides present in the CMV strains of the recipients were lacking. Consequently, a subgroup of recipients that were here considered to be at risk for rejection may, in fact, not belong to this group. More specifically, a recipient might have been misclassified when the mismatched peptide (i.e., the LIL or LLL peptide) was not the same peptide as the CMV UL40 peptide he could have generated an immune response against. Details on which peptide was present in the CMV strain by which the recipient was infected may significantly advance specification of the group who is at risk.

Potentially, the findings observed in this cohort could be due to a direct effect of the reported HLA allele groups. As such, we corrected for HLA compatibility between donor and recipient in our analyses. As a measure for HLA compatibility between donor and recipient, we evaluated PIRCHE-II scores instead of the number of mismatches, as PIRCHE-II provides a more gradual measure than calculating the number of HLA mismatches [33, 34]. Recipients receiving a donor mismatched for the one of the HLA peptides generally had higher PIRCHE-II scores compared to recipients without this mismatch. This difference was anticipated, as focusing on patients with a mismatched HLA class I leader peptide equals focusing on recipients mismatched for at least one of these alleles. Although this higher PIRCHE-II score may influence the risk of TCMR, a significant association remained after correction for the PIRCHE-II score between the LIL and LLL peptide mismatch and TCMR, indicating that the observed effect is not solely due to a higher number of available HLA-derived T-cell epitopes. Moreover, when comparing the peptide-mismatched recipients with and without CMV, PIRCHE-II scores were in the same range, indicating that the interaction with CMV is essential for the observed effects. Finally, the effect of an LIL or LLL leader peptide mismatch on TCMR was absent in the CMV-seronegative recipients, indicating that our findings are not solely due to the mismatched HLA alleles, but that the interaction with CMV is crucial.

In conclusion, we here for the first time show that a positive CMV serostatus of a transplant recipient combined with a LIL or LLL peptide mismatch strongly increases the risk of developing early TCMR after kidney transplantation, thereby representing an important risk factor for rejection. Preventing a leader peptide mismatch in these patients or adapting immunosuppression accordingly might decrease the incidence of TCMR early after transplantation.

Author Contributions

E.T.M.P. and E.S. conceptualised the idea for this study. R.d.M., F.M.V.L., J.M-.P., M.G-.D. and A.D.v.Z. facilitated the data collection and curation. E.T.M.P., R.d.M., J.J. and A.J.B. analysed the data. E.T.M.P., R.d.M., K.G., A.J.B., M.G-.D., A.D.v.Z. and E.S. were involved in the discussion and interpretations. All authors have critically revised the manuscript and approved the final version.

Conflicts of Interest

The UMC Utrecht has patents and patent applications on the prediction of an alloimmune response against mismatched HLA. ES is listed as inventor on these patents. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

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