THE ROLE OF IMMUNE CHECKPOINT MOLECULES IN SOLID AND HEMATOPOIETIC STEM CELL TRANSPLANTATION

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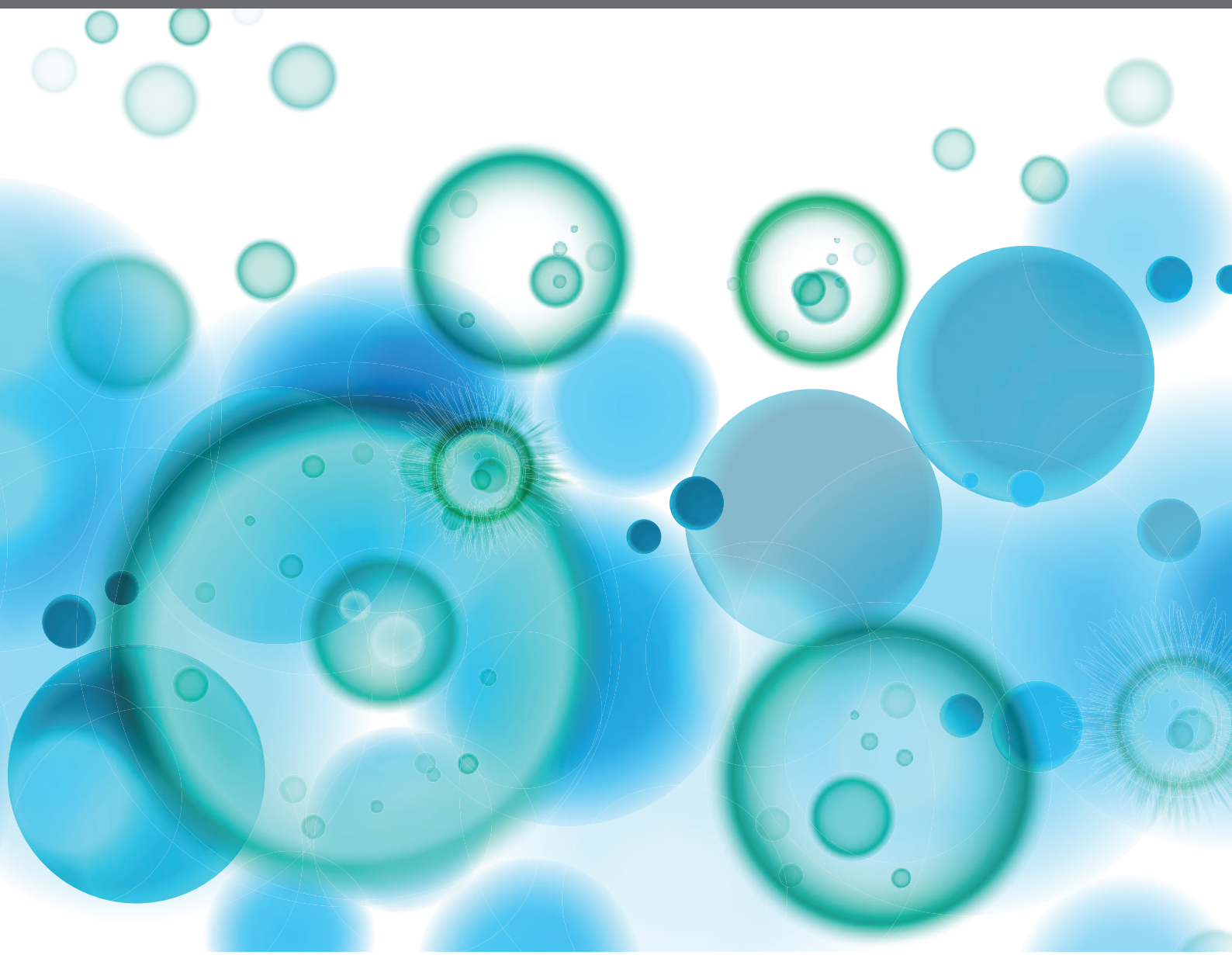

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THE ROLE OF IMMUNE CHECKPOINT MOLECULES IN SOLID AND HEMATOPOIETIC STEM CELL TRANSPLANTATION

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EDITORIAL
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Editorial: "The Role of Immune Checkpoint Molecules in Solid and Hematopoietic Stem Cell Transplantation"

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Keywords: immune checkpoint molecules, hematopoietic stem cell transplantation, solid transplantation, tolerance, HLA

Editorial to the Research Topic

The Role of Immune Checkpoint Molecules in Solid and Hematopoietic Stem Cell Transplantation

The success of both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) requires down-regulation of the allo-immune response. SOT and HSCT could only become standard therapies for many end-stage organ diseases or severe haematological malignancies thanks to the development of modern strategies, which aim at the suppression of the activity of T cells, B cells and NK cells recognizing genetic disparities between donor and recipient and as an ultimal goal the induction of tolerance. A proper downregulation of the alloimmune response is a prerequisite to prevent acute or chronic graft rejection in SOT and to avoid graft-versus-host disease (GvHD) in HSCT. However, keeping an equilibrium between a sufficient suppression of the allo-immune response or tolerance for the allo-antigens and maintenance of an adequate immune defense against infections and relapse continues to be a major challenge in transplantation.

Against this background, the role of immune checkpoints molecules (ICM) is of superior interest. Activation of ICM by interaction of co-inhibitory receptors with its cognate ligands is essential for maintaining immune homeostasis, diminishing tissue damage, and preventing unwanted autoimmunity. Dysregulation of ICM molecules can result in immune escape from host immune defense in infection and cancer. To further delineate these crucial pathways, a Research Topic was dedicated on *"The Role of Immune Checkpoint Molecules in Solid and Hematopoietic Stem Cell Transplantation"*.

Köhler et al. review the importance of ICM for relapse after allogeneic HSCT. They summarize that immune checkpoint blockade can increase anti-tumor immunity, but has been primarily successful in solid cancer therapy and Hodgkin lymphoma so far. Relapse after allogeneic HSCT is mainly thought to be attributable to loss of the graft-versus-leukemia (GVL) effect. One potential mechanism of immune escape from the GVL effect is the inhibition of allogeneic T cells *via* engagement of inhibitory receptors on their surface including PD-1, CTLA-4, TIM3, and others. This review provides an overview of current evidence for a role of immune checkpoint molecules for relapse and its treatment after allogeneic HSCT. The retrospective study of Mytilineos et al. analyses the influence of expression levels and mismatch permissiveness on the HLA-DPB1 degree of

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compatibility in the context of allogeneic HSCT. HLA-DPB1 mismatches can be classified in *permissive* and *non-permissive* mismatches by T-cell epitope matching. Non-permissive HLA-DPB1 mismatches showed significantly increased aGvHD risk if they were accompanied by two HLA-DPB1 mismatches in GvH direction or one mismatched highly expressed patient allotype. Non-permissive HLA-DPB1 mismatches is associated with a significantly higher risk of acute GvHD and non-relapse mortality. This study suggests that DP non-permissiveness associated with two HLA-DPB1 mismatches or at least on highly expressed mismatched patient allotype should be avoided. Kordelas et al. investigate the clinical significance of soluble PD-1 (sPD-1) after allogeneic HSCT regarding GvHD, relapse, and overall survival (OS) in a mono-centric cohort of 82 patients. They observed that low sPD-1 plasma levels at month one, two or three post HSCT were associated with acute GvHD grade III-IV, the onset of moderate/severe chronic GvHD and inferior OS, DFS, and TRM, respectively. Hence, this study pinpoints the soluble inhibitory co-receptor PD-1 as a promising candidate molecule for the prediction of clinical HSCT outcome. Chen et al. investigate whether the single-nucleotide polymorphisms (SNPs) of the co-stimulatory genes within non-HLA regions were related to the outcomes of allogeneic HSCT. Their results revealed that nine SNPs in the CTLA4 gene, five SNPs in the PDCD1 gene, two SNPs in the TNFSF4 gene, and four SNPs in the CD28 gene were significantly associated with the adverse outcomes following allogeneic HSCT. Duygu et al. review how NK cell alloreactivity and anti-viral immunity are regulated by NK cell receptors belonging to the KIR family and interacting with classical HLA class I molecules, or by NKG2A/C and LILRB1/KIR2DL4 engaging non-classical HLA-E or -G. Specifically, the authors focus on how NK cells contribute to the allo-immune response upon kidney transplantation either by promoting allograft rejection through lysis of cells of the transplanted organ or by promoting alloreactive T cells. Zhang et al. analyse the potential role of the novel immunosuppressant Belatacept for prevention of rejection following kidney transplant. To test the hypothesis that Belatacept combined with BTLA overexpression, may effectively attenuate acute rejection after kidney transplantation, the authors used a rat kidney transplantation model comparing graft rejection in single and combined therapy. By means of immunohistochemistry and flow cytometry, antigen-stimulated immune response by mixed lymphocyte culture, western blot and qRT-PCR analyses, the authors could show that Belatacept combined with BTLA overexpression attenuates acute rejection after kidney transplantation and prolonged kidney graft survival.

As up to now the key area of focus in ICM research and clinical implication has been in the field of cancer, this Research Topic highlights the contribution of ICM to allograft tolerance and to clinical outcome of SOT as well as to allogeneic HSCT.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of Interest: The 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.

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ORIGINAL RESEARCH
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The Human Leukocyte Antigen-DPB1 Degree of Compatibility Is Determined by Its Expression Level and Mismatch Permissiveness: A German Multicenter Analysis

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T-cell epitope matching according to the TCE3 algorithm classifies HLA-DPB1 mismatches in permissive and non-permissive. This classification has been shown to be predictive for mortality and acute GvHD (aGvHD) events in large international cohorts. We retrospectively genotyped HLA-DPB1 in 3523 patients transplanted in Germany between 2000 and 2014 and in their unrelated donors using an Illumina amplicon-NGS based assay. Aim of the study was to evaluate DP-compatibility beyond the established TCE3 algorithm by assessing the combined effect of several DP-mismatch parameters on post-transplant outcome. We implemented an extended DP-mismatch assessment model where TCE3, DP allotype expression with respect to rs9277534, mismatch vector and number of mismatches were conjointly taken into consideration. In this model, non-permissive HLA-DPB1 mismatches showed significantly increased aGvHD risk if they were accompanied by two HLA-DPB1 mismatches in GvH direction (HR: 1.46)

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or one mismatched highly expressed patient allotype (HR: 1.53). As previously reported, non-permissive HLA-DPB1 mismatches associated with a significantly higher risk of aGvHD and non-relapse mortality (HR 1.36 and 1.21, respectively), which in turn translated into worse GvHD and relapse free survival (HR 1.13). Effects on GvL and GvHD appeared strongest in GvH-directed non-permissive mismatches. Our study results support the consideration of additional HLA-DPB1 mismatch parameters along with the established TCE3 matching algorithm for refinement of future donor selection. In particular, our findings suggest that DP non-permissiveness associated with two HLA-DPB1 mismatches or at least on highly expressed mismatched patient allotype should be avoided.

Keywords: stem cell transplantation, graft-versus-host-disease, HLA-DPB1, HLA-DPB1 expression, HLA-DPB1-permissiveness

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation has become an established clinical treatment for various, otherwise often incurable diseases of the lympho-hematopoietic system. Improvements in treatment protocols as well as donor selection procedures have led to increasing numbers of patients undergoing hematopoietic stem cell transplantation (HSCT) (1). Although the first choice is usually an HLA-identical sibling, often such donors are not available and therefore unrelated donors are used (2). As the segregation of haplotypes in unrelated donors cannot be determined, only locus-wise matching is performed and depending on the frequency of the patient's HLA-phenotype, sometimes HLA-differences have to be accepted (3). It has become apparent, that matching for the antigen recognition domain (ARD) for classical HLA-loci improves post-transplant mortality and morbidity (4). The minimal consensus on compatibility testing requires high resolution typing for HLA-A, -B, -C, and -DRB1. Many centers in Europe also include HLA-DQB1 compatibility in donor selection strategies. The relevance of HLA-DPB1 matching in unrelated stem cell transplantation has long remained undefined. This may be due to several characteristics that distinguish HLA-DPB1 antigens from other classical HLA-molecules. First, HLA-class II molecules are formed as heterodimers of an alpha and a beta chain; the ARD is formed by the alpha-1 and the beta-1 domain. Most polymorphisms are located within the beta-1 domain (exon 2 of the respective gene). These polymorphisms are almost evenly distributed across allotypes of classical HLA-molecules. In contrast, for HLA-DPB1, most of the polymorphisms are observed within six polymorphic regions throughout exon 2 of the HLA-DPB1 gene, resulting in several clusters with similar immunogenicity (5). This leads to significantly less diversity regarding T-cell epitopes. Second, the linkage disequilibrium between classical HLA-genes is very strong, particularly for HLA-B/C and HLA-DR/DQ genes, forming conserved haplotypes, which are frequently observed (6). However, the linkage between HLA-DPB1 and other classical HLA-genes is rather low because of a recombination hotspot between the HLA-DQ and HLA-DP genes, which in turn occasionally leads to HLA-DPB1 disparities among apparently HLA-identical sibling donors and far more often to HLA-DPB1 incompatibility between recipients and their otherwise fully HLA-matched unrelated donors (~80%) (7, 8). Third, the expression of HLA-DPB1 is similar to that of HLA-DRB3/4/5 and HLA-DQB1 antigens and lower as compared to the classical HLA-antigens HLA-A, -B, -C, and -DRB1 (9, 10). The former are therefore referred to as low expression loci (LEL) and the latter as high expression loci (HEL). Last, serological typing for HLA-DPB1 has always been much more difficult due to lack of suitable antisera. It has been shown that only two sets of dimorphic amino acid epitopes account for most of the serological reaction patterns observed, resulting in considerably less diversity compared to the other classical HLA-antigens (11).

Early studies had shown that the impact of HLA-DPB1 differences on the incidence of GvHD was balanced by a lower relapse rate and therefore did not translate into better survival outcomes (12). It was also recognized that HLA-DPB1 differences might have an additional detrimental effect on the presence of other mismatches. Later it was discovered using cytotoxicity assays that HLA-DPB1 alleles may be grouped according to their T-cell immunogenicity into three groups (13). This led to the T-cell epitope matching algorithm, which allows grouping of DP-mismatches between patient and donor in permissive and non-permissive and which has been shown to associate with clinical outcome in large retrospective cohorts (7, 14). Another proposed model relates to the expression levels of HLA-DPB1 mismatches, which is influenced by an SNP in the 3'-UTR of HLA-DPB1 alleles (rs9277534) (15). Aim of our study was to validate these models in an independent cohort and to explore, if the two models are possibly complementary.

PATIENTS AND METHODS

Study Cohort

This study included patients transplanted for various hematological diseases with peripheral blood stem cells (PBSC) or bone marrow (BM) from an unrelated donor at German

centers. The transplants were performed from 2000 to 2014. All searches were conducted by the search unit in Ulm. Only transplants with first allogeneic transplantation were included. Disease status at time of transplantation was classified according to the definitions used in the establishment of the EBMT risk score (16). Myeloablative conditioning (MAC) was classified according to the definitions for standard intensity conditioning regimens of the EBMT MED-AB manual Appendix III and published consensus suggestions (17). Less intense regimens were considered as reduced intensity (RIC). Most of the patients received in-vivo T-cell depletion with ATG or Campath. Standard of post-Tx immunosuppression was a cyclosporine based treatment approach in the vast majority of cases. Study design, collection of clinical data and ethics aspects are described in detail in the **Supplemental Material**.

HLA-Typing

For all patients high resolution HLA-typing was available for the gene loci HLA-A, -B, -C, -DRB1 and -DQB1, defining all polymorphisms within the ARD – exons 2 and 3 for HLA-class I, and exon 2 for HLA-class II molecules (18). Non-expressed alleles were excluded according to NMDP confirmatory typing requirements. For HLA-DPB1 retrospective typing was applied based on an NGS-amplicon sequencing methodology using the Illumina (San Diego, CA, USA) platform. This in-house protocol was validated and CE-certified as IVD-reagent and is routinely used in stem cell donor typing. HLA-alleles are considered as matched if they show the same protein sequence within the ARD.

Definitions

HLA-DPB1 TCE3 matching was performed according to the revised TCE3 matching procedure based on functional distance (19). DPB1 mismatches were classified as permissive and non-permissive. In some models for non-permissive mismatches mismatch directionality (i.e. GvH vs HvG) was considered. Prediction of SNP rs9277534 was based on HLA-DPB1 genotyping using imputed information as previously described (20). With respect to rs9277534, DPB1 mismatches were categorized into two surface expression groups (G allele as high and A allele as low expressed). In the combined DP mismatch model, TCE3, rs9277534, mismatch vector as well as number of mismatches were conjointly taken into consideration. Specifically, for the expression part only mismatched allotypes in GvH direction (the mismatched patient allotype) were considered ranging from matched to zero mismatches in GvH vector, one mismatch in GvH vector and "low-expressed" (rs9277534-A), one mismatch in GvH vector and "high expressed"(rs9277534-G) and both mismatched alleles irrespective of rs9277524 genotype. For the immunogenicity part the hierarchy with increasing risk was DP matched, DP permissive mismatched and DP non-permissive mismatched. As to the overall number of DP mismatches, this was calculated on the basis of GvH direction only.

Endpoints of interest were overall survival (OS), GvHD and relapse-free survival (GRFS), non-relapse mortality (NRM), aGvHD incidence and relapse incidence. OS was defined as time to death or last follow-up. GRFS was defined as time to aGvHD, relapse or death, whichever occurred first. NRM was defined as time to death from any cause except relapse. A relapse event was treated as competing risk. The endpoint aGvHD incidence was defined as time to first diagnosis of aGvHD (grades II-IV). An additional subanalysis for aGvHD (grades III-IV) was conducted. Death from any cause without prior aGvHD was considered as competing risk. Relapse incidence was defined as time to relapse and death from any cause without prior relapse was treated as competing risk. Patients alive and/or free from the event of interest were censored at last follow-up (21).

Statistical Analysis

For descriptive statistics, the chi-squared test was used for categorial variables and the Mann-Whitney-U-Test for continuous variables. For survival analyses of the endpoints OS and GRFS Kaplan-Meier estimates were used and comparisons were performed with the log-rank test (22). For the endpoints NRM, aGvHD and relapse, cumulative incidence curves for competing risk data were generated and compared with the method of Gray (23). For multivariate analyses cause specific Cox models have been used, allowing for adjustment of time-dependent covariate effects in a piecewise constant manner (24). The breakpoints were chosen graphically (22). A center effect was adjusted. As this study represents a validation study of previous analyses, a significance level of 0.05 was considered sufficient for confirmation.

RESULTS

High Prevalence of HLA-DPB1 Mismatches in 10/10 HSCT 9/10 HLA-Matched Hematopoietic Stem Cell Transplantation

The cohort consisted of 10/10 (n=2450, 69.5%) and 9/10 HLA (i.e. HLA-A, -B, -C, -DRB1, -DQB1) matched transplant pairs (n=1073, 30.5%). The distribution of diagnoses was similar in both groups, median age was slightly lower in the 9/10 matched transplants. Details regarding the cohort's features are shown in **Table 1**. Median follow-up was 52 months.

Retrospective genotyping of HLA-DPB1 locus in patients and their respective donors confirmed the high prevalence of HLA-DPB1 mismatches in both, 10/10 and 9/10 HLA-matched transplantations already reported elsewhere (7, 25, 26). Specifically, in the subgroup of 10/10 HLA-matched transplantations only 21.3% (n=521) were HLA-DP identical, while in the subgroup of 9/10 HLA-matched this fraction was 18.5% (n=198). Further categorization of DP mismatches as to permissiveness according to TCE3 revealed that in 37.9% (n=929) of 10/10 and in 34.9% (n=375) of 9/10 matched transplantations, respectively, the DP mismatch was permissive. For the remainder of the transplantations the HLA-DPB1 mismatches were non-permissive with even sub-

TABLE 1 | Patient characteristics.

	10/10 (%)	9/10 (%)	Total	P-Value
N	2450	1073	3523	n.a.
Median age (range)	54 (0–77)	52 (0–76)	53 (0–77)	0.014
AML	852 (34.8)	401 (37.4)	1253	0.466
MDS	375 (15.3)	161 (15.0)	536	
NHL	313 (12.8)	114 (10.6)	427	
ALL	273 (11.1)	131 (12.2)	404	
Myeloma	231 (9.4)	94 (8.8)	325	
CLL	135 (5.5)	54 (5.0)	189	
Acute Leukemia	120 (4.9)	45 (4.2)	165	
other	79 (3.2)	34 (3.2)	113	
CML	72 (2.9)	39 (3.6)	111	
Early stage	924 (37.7)	420 (39.1)	1344	0.531
Intermediate stage	837 (34.2)	346 (32.2)	1183	
Advanced stage	629 (25.7)	280 (26.1)	909	
Unknown or n.a.	60 (2.4)	27 (2.5)	87	
KPS 80-100	1,875 (76.5)	740 (69.0)	2615	0.119
KPS <80	116 (4.7)	60 (5.6)	176	
Missing	459 (18.7)	273 (25.4)	732	
BM	146 (6.0)	85 (7.9)	231	0.036
PBSC	2,304 (94.0)	987 (92.0)	3291	
Missing	0 (0)	1 (0.1)	1	
MAC	1,501 (61.3)	699 (65.1)	2200	**0.033**
RIC	948 (38.7)	374 (34.9)	1322	
Missing	1 (0)	0 (0)	1	
ATG/Campath	1,652 (67.4)	701 (65.3)	2353	0.400
No ATG/Campath	514 (21.0)	193 (18.0)	707	
Missing	284 (11.6)	179 (16.7)	463	
Donor Age 18-30	845 (34.5)	309 (28.8)	1154	**<0.001**
Donor Age 31-45	1,174 (47.9)	480 (44.7)	1654	
Donor age 46-60	363 (14.8)	211 (19.7)	574	
Missing	68 (2.8)	73 (6.8)	141	
P-D CMV neg neg	787 (32.1)	299 (27.9)	1086	**<0.001**
P-D CMV neg pos	205 (8.4)	111 (10.3)	316	
P-D CMV pos neg	569 (23.2)	306 (28.5)	875	
P-D CMV pos pos	760 (31.0)	294 (27.4)	1054	
Missing	129 (5.3)	63 (5.9)	192	
P-D ABO match	995 (40.6)	418 (39)	1413	0.421
P-D ABO major	551 (22.5)	249 (23.2)	800	
P-D ABO bidir	223 (9.1)	115 (10.7)	338	
P-D ABO minor	613 (25.0)	262 (24.4)	875	
Missing	68 (2.8)	29 (2.7)	97	

Acute Leukemia, undifferentiated, biphenotypic, secondary or unclassified; n.a., not applicable; KPS, Karnofsky performance score; BM, bone marrow; PBSC, peripheral blood stem cells; MAC, myeloablative conditioning; RIC, reduced intensity conditioning; P-D Patient-Donor; major, major incompatibility; bidir, bidirectional incompatibility; minor, minor incompatibility.

distributions into the GvH and HvG vector. Almost half of the transplantations were single DP-mismatches, while 30% showed two DP differences. A double DP mismatch in GvH direction regardless of permissiveness was seen in about 23% of the cases. These data are summarized in **Table 2**. Additional multivariate analyses considering separately 10/10 and 9/10 HLA matched cases showed that the HLA-DP mismatch effect remained constant and uninfluenced by the presence of an additional HLA mismatch with the exception of relapse. The latter is analyzed in more detail right after. The data of these analyses are presented in detail in the **Supplemental Material** [**S5-S7, Supp3-Supp7(F)**].

TABLE 2 | Results of HLA-DPB1 TCE3 matching.

	10/10 (%)	9/10 (%)	Total
DP matched	521 (21.3)	198 (18.5)	719
DP permissive MM	929 (37.9)	375 (34.9)	1304
DP non-permissive GvH vector	493 (20.1)	241 (22.5)	734
DP non-permissive HvG vector	507 (20.7)	259 (24.1)	766
DP non-permissive MM total	1,000 (40.8)	500 (46.6)	1,500
DP 1 MM	1,206 (49.2)	522 (48.6)	1,728
DP 2 MM	723 (29.5)	353 (32.9)	1,076
DP non-permissive 2MM GvH vector	377 (15.4)	196 (18.2)	573
DP permissive 2MM GvH vector	165 (6.7)	70 (6.5)	503

MM, Mismatch; GvH, Graft versus Host; HvG, Host versus Graft.

Known Associations of Non-Permissive HLA-DPB1 Mismatches With Outcome Endpoints Confirmed

Regarding the effect of HLA-DPB1 mismatch on outcome endpoints, our results are in line with those previously reported. Specifically as to GRFS, 10/10 HLA-matched transplant pairs with HLA-DP non-permissive mismatches compared to HLA-DP matched or permissively mismatched cases exhibited a clearly higher composite risk of relapse, GvHD II-IV or death as presented in the GRFS outcome endpoint. These results are graphically depicted in **Figure 1** (p=0.002) and in more detail presented in **Table 3**. A similar result was seen in the subgroup of 9/10 matched transplantations where also patients with a DP matched or permissively mismatched donor showed significantly better GRFS (p=0.026), (**Figure 2**, **Table 3**). Multivariate analysis confirmed the results of the univariate models with non-permissive DP mismatches associating with significantly inferior GRFS (HR 1.16, CI 1.08–1.26, p<0.001, **Table 4**). No significant difference was observed between TCE3 permissively mismatched and fully DP matched transplantations with regard to this endpoint (HR 0.95, CI 0.86–1.06, p=0.401). Another GRFS analysis considering GvHD III-IV led to similar findings (**Table S2** in Supplemental material). It is of note that in the composite endpoint GRFS, the impact of TCE3 matching showed a time-dependent effect for non-permissive mismatches. This effect was only significant in the first 100 days after transplantation (non-permissive until d100: HR 1.23, CI 1.11-1.36, p<0.001 and non-permissive GvH direction until d100: HR 1.31, CI 1.16–1.48, p<0.001). Afterwards, non-permissive mismatches showed only a non-significantly increased risk of worse GRFS (**Table S1** in Supplemental Material). Time-dependent effects of other clinical covariates were also modeled and are shown in **Table S1** in Supplemental Material. These time-dependent covariables resemble effects that were explored and published previously (24, 27).

Non-relapse mortality was also significantly higher in DP non-permissive mismatched transplant pairs, both in the 10/10 HLA-matched (p=0.010, **Figure 3A**) and in the 9/10 HLA-matched group (p=0.013, **Figure 3B**). The results of the univariate analyses are shown in detail in **Table 3**. Again, the results of the univariate analysis were confirmed in the multivariate models, as non-permissive mismatches showed a

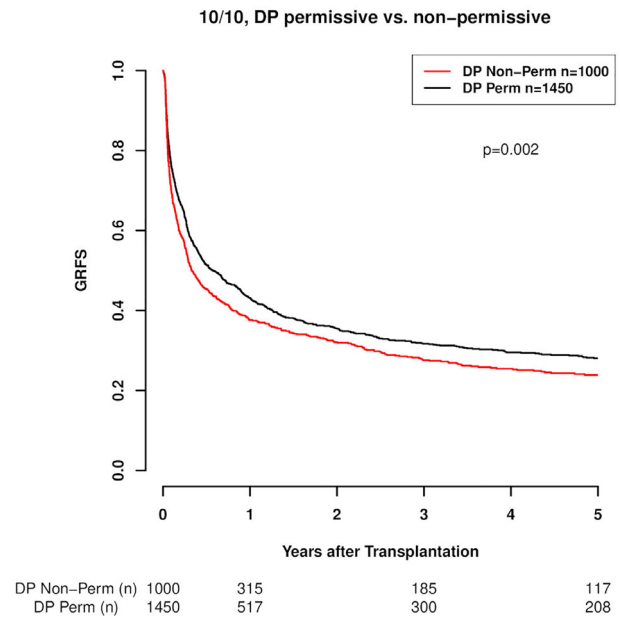

FIGURE 1 | GRFS (Graft vs Host disease- and relapse-free survival; GvHD II-IV) in 10/10 HLA-matched cases with respect to DP mismatch permissiveness according to TCE3. DP Perm vs. DP Non-Perm cases, where DP Perm = DP matched or TCE3 permissively mismatched and DP Non-Perm = DP TCE3 non-permissively mismatched (p=0.002).

significantly higher risk of NRM when compared to DP matched transplantations (HR 1.21. CI 1.02–1.42, p=0.029, **Table 4**). Permissive mismatches showed a risk similar to DP matched transplantations (HR 0.97, CI 0.81–1.15, p=0.693), as seen for GRFS. As expected the incidences of aGvHD were significantly higher in the HLA-DPB1 non-permissive mismatched groups in both univariate and multivariate models (**Tables 3** and **4**, **Figures 3C, D**). Analysis of the effect of HLA-DP MM on the incidence of chronic GvHD did not show significant results. The findings of this analysis are presented in the supplemental material (**Table S7**).As far as relapse incidence is concerned, both univariate and multivariate analyses clearly showed a significantly lower risk for HLA-DP non-permissive mismatches in otherwise 10/10 HLA-matched transplantations (p=0.045, **Figure 3E**). The respective results are shown in detail in **Tables 3** and **4**. Multivariate analysis of relapse incidence in patients with advanced disease stage confirmed the results of the whole cohort as to the effect of non-permissive DP mismatches (**S3** in **Supplemental Material**). Additional subanalyses comparing the DP-matched group separately vs. the TCE3 permissively and non-permissively mismatched group, respectively in 10/10 and 9/10 HLA matched transplantations revealed that DP matched cases exhibit a significantly higher risk of relapse compared to both, TCE3 permissively and non-permissively mismatched cases (HR 0.85, CI 0.73-0.99), p=0.038 for DP matched vs. TCE3 permissive mismatched; HR 0.81, CI 0.69-0.94), p=0.006 for DP matched vs. TCE3 non-permissively mismatched in 10/10 HLA transplantations). This was however, evident only in the 10/10 HLA matched setting, as the additional HLA mismatch appeared to completely abrogate that beneficial effect of DP mismatch (both permissive and non-permissive) vs. DP match as to lower relapse incidence. This was seen in both, multivariate and univariate models for relapse incidence. These data are presented in more detail in the supplemental material **(Supp7(E), Supp7(F), S5** and **S6**). Furthermore the impact of DP mismatches on OS was not statistically significant in either univariate (data not shown) or multivariate analyses (**Table 4**). Last, a significant impact of CMV matching status on transplantation outcome was seen neither in the 10/10 nor the 9/10 HLA-matched group (**Table S5** and **S6**).

Mismatch Directionality Relevant Only in aGvHD and Relapse

Subanalysis of the vector of non-permissiveness against DP matched/permissively mismatched showed significantly higher risks for both GvH and HvG directed mismatches in the GRFS endpoint. (Non-permissive GvH: HR 1.19, 1.08–1.31, p<0.001 and non-permissive HvG: HR 1.14, 1.03–1.26, p=0.001). The detrimental effect of the non-permissive mismatches on NRM was again independent of the mismatch directionality as both, GvH and HvG vector non-permissive mismatches, associated with increased NRM risk (GvH: HR 1.20, CI 1.03–1.41, p=0.014; HvG: HR 1.26, CI 1.09–1.46, p=0.002). Contrary to the previous endpoint analyses, the mismatch vector appeared to indeed play a role in aGvHD incidence, as the higher risk observed was mostly driven by non-permissive mismatches in GvH direction (HR 1.50, CI 1.29–1.75, p<0.001). In line with the results for aGvHD, the effect of non-permissive mismatches on relapse incidence appeared to be mainly driven by the GvH vector (GvH direction: HR 0.84, CI 0.73–0.97, p=0.018; HvG direction: HR 0.98, CI 0.86–1.12, p=.0763; **Table 4**). It is of note that this vector effect was not seen in the subanalysis of patients with advanced disease, as no differences were seen between GvH and HvG vectors (**Table S3** in Supplemental Material).

Effect of Non-Permissive HLA-DPB1 Mismatch Aggravated by Increasing Number of DP Mismatches and High Expression Level of Mismatched Allotype in GvH Vector

In the combined DP mismatch (TCE3-rs9277534) model, an interesting finding was that apart from TCE3 permissiveness also the overall number of DP mismatches as well as the anticipated expression level of the mismatched allotype with a GvH vector contributed to the overall mismatch effect. Specifically, a significantly higher risk of GRFS, NRM and aGvHD was found for DP non-permissive mismatches with two overall DP-allele-mismatches in GvH direction (**Table 5**). For aGvHD incidence, also DP non-permissive mismatches with one high expressed mismatched patient allotype (rs9277534-G) showed significantly higher risk estimates (**Table 5**). Conversely, with respect to relapse incidence, these categories associated with significantly lower risk as shown in **Table 5**. The enhancement of non-permissive DP effect on the aforementioned endpoints becomes clear after comparison of

TABLE 3 | Univariate analysis.

	Univariate Analysis					
	10/10 HLA-matched HSCT			9/10 HLA-matched HSCT		
Endpoints	**HLA-DP matched/permissive MM**	**HLA-DP non-permissive MM**	**p-value**	**HLA-DP matched/permissive MM**	**HLA-DP non-permissive MM**	**p-value**
GRFS (GvHD II-IV)						
1 year	43.1% (40.5–45.9)	37.6% (34.6–40.9)		34.4% (30.5–38.7)	28.8% (24.9–33.2)	
3 year	31.7% (29.1–34.4)	27.6% (24.6–30.8)	**0.002**	25.7% (22.0–29.9)	20.1% (16.6–24.3)	**0.026**
5 year	28.0% (25.5–30.8)	23.9% (21.0–27.2)		21.9% (18.4–26.2)	18.1% (14.7–22.3)	
GRFS (GvHD III–IV)						
1 year	49.1% (46.5–51.9)	45.3% (42.2–48.7)		39.7% (35.8–44.1)	38.1% (35.8–44.1)	
3 year	34.4% (31.8–37.1)	31.9% (28.8–35.2)	**0.014**	29.6% (25.9–33.9)	25.2% (21.5–29.6)	0.191
5 year	29.0% (26.5–31.8)	27.5% (24.6–30.8)		23.3% (19.7–27.5)	21.8% (18.2–26.1)	
Non-relapse mortality						
1 year	20.9% (18.7–23.1)	25.6% (22.8–28.5)		27.0% (23.3–30.9)	33.1% (28.8–37.5)	
3 year	26.2% (23.8–28.7)	31.0% (27.9–34.1)	**0.010**	31.6% (27.6–35.7)	40.0% (35.3–44.6)	**0.013**
5 year	27.6% (25.1–30.2)	32.7% (29.6–36.0)		33.7% (29.5–38.0)	42.0% (37.2–46.7)	
Relapse incidence						
1 year	28.3% (25.9–30.8)	25.8% (23.0–28.7)		27.6% (23.5–31.8)	29.9% (26.0–33.9)	
3 year	38.5% (35.8–41.3)	34.3% (31.0–37.5)	**0.045**	36.2% (31.6–40.8)	37.3% (33.0–41.5)	0.201
5 year	41.9% (39.1–44.8)	37.0% (33.6–40.3)		38.4% (33.6–43.1)	41.8% (37.3–46.3)	
aGvHD II–IV incidence						
at day 100 after HSCT	22.9% (20.8–25.1)	29.4% (26.6–32.4)	**<0.001**	31.3% (27.5–35.2)	38.4% (34.1–42.6)	**0.010**

GRFS, GvHD and relapse free survival; GvHD, Graft versus Host Disease; MM, mismatch; HSCT, Hematopoietic Stem Cell Transplantation; HLA, Human Leukocyte Antigen. Statistical significance marked in bold.

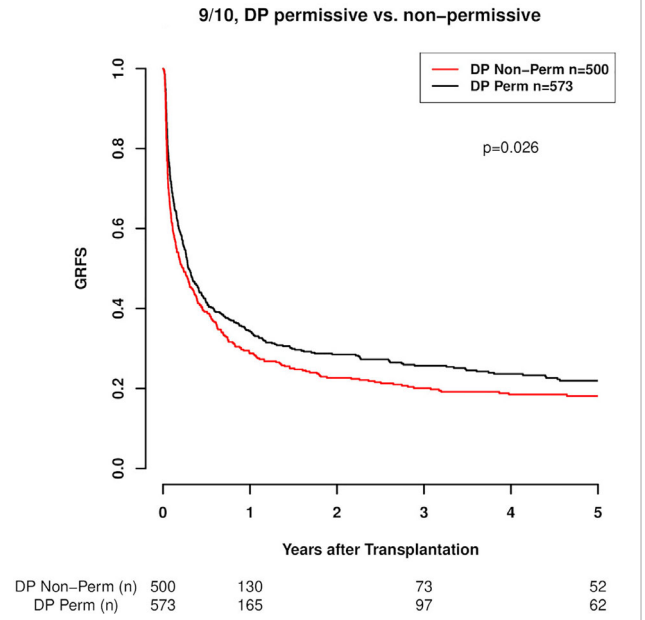

FIGURE 2 | GRFS (Graft vs Host disease- and relapse-free survival; GvHD II-IV) in 9/10 HLA-matched cases with respect to DP mismatch permissiveness according to TCE3. DP Perm vs. DP Non-Perm cases, where DP Perm = DP matched or TCE3 permissively mismatched and DP Non-Perm = DP TCE3 non-permissively mismatched (p=0.026).

the respective hazard risks for non-permissive DP mismatches overall and for non-permissive DP mismatches with highly expressed patient mismatched allotype or double mismatch in GvH direction as presented in **Tables 4** and **5**. Although statistical significance was not reached in the subgroup of two overall permissive mismatches in GvH direction, a clear trend was seen at least for aGvHD (**Table 5**).

DISCUSSION

Several factors seem to contribute to the alloreactivity is induced by HLA-DPB1 differences. These are linked to the intrinsic immunogenicity on account of T-cell epitopes, the numbers and vectors of mismatches as well as the expression level of the mismatched allele. HLA-DPB1 mismatching represents therefore a multilevel variable and any individual model represents only a simplification of the true biological relationship. In this retrospective study we sought to conjointly assess the effect of the aforementioned factors with the aim to offer a more unified approach as to DP mismatch evaluation for donor selection. Through our analysis we were able to confirm previously described associations, while we also showed that consideration of additional factors might be meaningful for histocompatibility assessment and for improving predictiveness.

Regarding the prevalence of HLA-DPB1 mismatches in 10/10 as well as 9/10 HLA-matched HSCTs, no differences were

TABLE 4 | Multivariate analysis.

	OS		GRFS		NRM		Relapse		aGVHD II-IV	
	HR (CI)	**P-value**	**HR (CI)**	**P-value**	**HR (CI)**	**P-value**	**HR (CI)**	**P-value**	**HR (CI)**	**P-value**
Patient Age	1.02 (1.01–1.02)	**<0.001**	1.01 (1.01–1.01)*	**<0.001**	1.02 (1.02–1.03)	**<0.001**	1.01 (1.00–1.01)	**0.004**	–	
Early stage disease	1.00		1.00		1.00		1.00		–	
Intermediate stage disease	1.23 (1.08–1.40)	**0.002**	1.23 (1.11–1.37)	**<0.001**	1.02 (0.87–1.20)	0.816	1.66 (1.43–1.93)	**<0.001**	–	
Advanced disease stage	1.85 (1.63– 2.10)*	**<0.001**	1.53 (1.38–1.70)	**<0.001**	1.31 (1.13–1.53)*	**<0.001**	2.06 (1.77–2.38)*	**<0.001**	–	
10/10 HLA	1.00		1.00		1.00		–		1.00	
9/10 HLA	1.26 (1.14–1.40)	**<0.001**	1.26 (1.16–1.37)	**<0.001**	1.28 (1.12–1.46)	**<0.001**	–		1.41 (1.24–1.61)	**<0.001**
Patient HLA-C KIR Ligand group C1x	1.00		–		–		–		–	
Patient HLA-C KIR Ligand group C2C2	1.18 (1.04–1.35)	**0.012**	–		–		–		–	
Donor age 18–30	1.00		1.00		1.00		–		1.00	
Donor age 31–45	1.13 (1.01–1.27)	**0.029**	1.08 (0.99–1.19)	0.085	1.18 (1.02–1.37)	**0.028**	–		1.17 (1.01–1.35)	**0.037**
Donor age 46–60	1.25 (1.08–1.45)	**0.003**	1.12 (0.99–1.26)	0.067	1.48 (1.24–1.78)	**<0.001**	–		1.25 (1.04–1.51)	**0.017**
P-D CMV neg-neg	1.00		–		–		–		–	
P-D CMV neg-pos	1.13 (0.94–1.36)	0.190	–		–		–		–	
P-D CMV pos neg	1.14 (0.99–1.30)	0.061	–		–		–		–	
P-D CMV pos pos	1.09 (0.95–1.23)	0.210	–		–		–		–	
RIC	1.00		1.00		1.00		–		–	
MAC	1.23 (1.08–1.39)*	**0.001**	1.15 (1.04–1.27)	**0.007**	1.28 (1.11–1.47)*	**<0.001**	–		–	
KPS 80–100	1.00		1.00		–		1.00		–	
KPS <80	1.56 (1.26–1.93)	**<0.001**	1.37 (1.15–1.64)	**0.001**	–		1.38 (1.09–1.76)*	**0.007**	–	
No in-vivo T-cell depletion	1.00		1.00		–		1.00		1.00	
***In vivo* T-cell depletion**	0.84 (0.72–0.97)*	**0.015**	0.78 (0.69–0.88)*	**<0.001**	–		0.80 (0.69–0.92)	**<0.001**	0.67 (0.58–0.78)	**<0.001**
Year of Tx 2000–2003	–		1.00		1.00		1.00		1.00	
Year of Tx 2004–2009	–		0.56 (0.42–0.76)	**<0.001**	0.58 (0.40–0.84)	**0.005**	1.88 (1.10–3.24)	**0.022**	0.56 (0.37–0.84)	**0.005**
Year of Tx 2010–2014	–		0.57 (0.41–0.78)	**<0.001**	0.57 (0.39–0.84)	**0.004**	1.93 (1.11–3.34)	**0.020**	0.57 (0.37–0.86)	**0.008**
TCE3 Permissive/DP matched	1.00		1.00		1.00		1.00		1.00	
TCE3 Non-permissive	1.03 (0.94–1.14)	0.543	1.16 (1.08–1.26)*	**<0.001**	1.23 (1.09–1.39)	**<0.001**	0.90 (0.81–1.01)	0.094	1.33 (1.17–1.51)	**<0.001**
DP matched	1.00		1.00		1.00		1.00		1.00	
TCE3 permissive	0.91 (0.80–1.04)	0.179	0.95 (0.86–1.06)	0.401	0.97 (0.81–1.15)	0.693	0.91 (0.78–1.05)	0.198	1.04 (0.86–1.24)	0.698
TCE3 Non-permissive	0.97 (0.85–1.11)	0.662	1.13 (1.02–1.26)*	**0.025**	1.21 (1.02–1.42)	**0.029**	0.85 (0.74–0.99)	**0.033**	1.36 (1.15–1.61)	**<0.001**
TCE3 Permissive/DP matched	1.00		1.00		1.00		1.00		1.00	
TCE3 Non-permissive GvH	0.95 (0.84–1.08)	0.455	1.19 (1.08–1.31)*	**<0.001**	1.20 (1.03–1.41)	**0.014**	0.84 (0.73–0.97)	**0.018**	1.50 (1.29–1.75)	**<0.001**
TCE3 Non-permissive HvG	1.11 (0.98–1.24)	0.093	1.14 (1.03–1.26)	**0.001**	1.26 (1.09–1.46)	**0.002**	0.98 (0.85–1.12)	0.763	1.18 (1.01–1.38)	**0.037**

P-D, Patient-Donor; RIC, Reduced intensity conditioning,; MAC, Myeloablative conditioning; TCE3, T-cell epitope 3 matching; HR, Hazard ratio; CI, Confidence interval. Covariates showing time-dependent effects are labeled with an asterisk (*), -, not included in model. Statistical significance marked in bold.

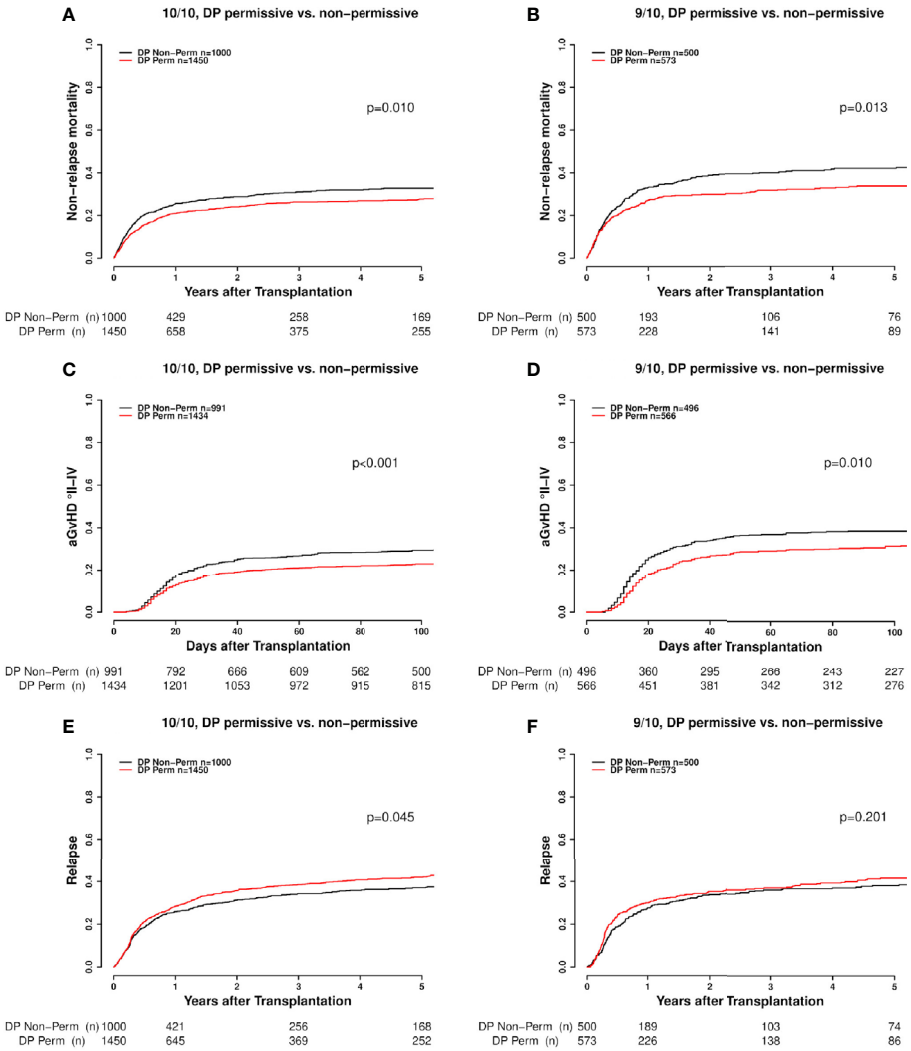

FIGURE 3 | **(A–F)** Competing risks outcomes [non-relapse mortality (NRM), acute GvHD (aGvHD) and relapse] with respect to DP mismatch permissiveness according to TCE3 in 10/10 and 9/10 HLA-matched cases. DP Perm vs. DP Non-Perm cases, where DP Perm = DP matched or TCE3 permissively mismatched and DP Non-Perm = DP TCE3 non-permissively mismatched.

observed between our findings and those seen in other studies (7, 25, 26). The same applies for distribution of non-permissive mismatches in GvH and HvG direction, which was balanced in the respective immunogenicity models (28). HLA-DP matched and permissively mismatched transplants have been grouped together for this analysis, as the broadly used TCE algorithm tool makes no distinction between these two groups for which we only observed a difference in the relapse analysis as already mentioned before.

In line with previously reported associations of HLA-DPB1 non-permissive mismatches with outcome endpoints, we also observed a clearly higher risk of aGvHD and NRM (7, 14). Although the risk of relapse was significantly lower in the non-permissive mismatch group, the composite GRFS endpoint was overall inferior compared to the matched/permissively mismatched ones, most probably due to the detrimental effect of non-permissive HLA-DPB1 mismatches on aGvHD incidence. The higher induced T-cell alloreactivity most likely accounts for this effect, as this has been previously shown by in-vitro testing (5, 13). Interestingly, with respect to OS, the two opposite effects of NRM and relapse appear to have mutually eliminated one another, as no significant differences were observed with respect to DP mismatch permissiveness. Closer look into the death cause analysis in DP non-permissively mismatched and matched/permissively mismatched cases may explain the aforementioned observation on OS (death cause analysis results are presented in detail in the **Supplemental Material** section). Although non-permissive HLA-DPB1

TABLE 5 | Combined DP-mismatch model.

	OS		GRFS		NRM		Relapse		aGVHD II-IV	
	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value	HR (CI)	P-value
DP matched, N=719	1.00		1.00		1.00		1.00		1.00	
DP Permissive MM, 0MM GvH, N=6	0.80 (0.63–1.01)	0.060	0.78 (0.29–2.10)	0.622	0.60 (0.08–4.26)	0.605	0.69 (0.17–2.78)	0.602	0.72 (0.10–5.14)	0.742
DP Permissive MM, 1MM GvH (A), N=742	0.49 (0.12–1.97)	0.312	0.91 (0.80–1.03)	0.119	0.92 (0.75–1.13)	0.433	0.88 (0.74–1.04)	0.122	0.94 (0.76–1.16)	0.551
DP Permissive MM, 1MM GvH (G), N=321	0.88 (0.76–1.03)	0.105	0.98 (0.84–1.16)	0.803	0.99 (0.77–1.27)	0.949	0.94 (0.77–1.16)	0.592	1.08 (0.83–1.40)	0.561
DP Permissive MM, 2MM GvH, N=235	0.92 (0.76–1.11)	0.360	1.17 (0.94–1.33)	0.215	1.10 (0.83–1.44)	0.515	0.97 (0.77–1.23)	0.803	1.30 (0.98–1.71)	0.065
DP Non-Permissive MM, 0MM GvH, N=125	1.02 (0.83–1.26)	0.825	1.01 (0.81–1.27)	0.916	1.09 (0.76–1.54)	0.648	1.14 (0.85–1.54)	0.371	0.93 (0.63–1.38)	0.721
DP Non-Permissive MM, 1MM GvH (A), N=365	1.07 (0.82–1.39)	0.641	1.05 (0.90–1.21)	0.563	1.08 (0.86–1.38)	0.494	1.02 (0.84–1.25)	0.831	1.15 (0.90–1.46)	0.264
DP Non-Permissive MM, 1MM GvH (G), N=437	0.93 (0.78–1.11)	0.428	1.13 (0.98–1.30)	0.094	1.16 (0.93–1.45)	0.187	0.76 (0.62–0.94)	**0.010**	1.53 (1.23–1.91)	**<0.001**
DP Non-Permissive MM, 2MM GvH, N=573	0.92 (0.77–1.09)	0.341	1.22 (1.07–1.39)	**0.002**	1.37 (1.13–1.67)	**0.002**	0.76 (0.63–0.92)	**0.004**	1.46 (1.19–1.79)	**<0.001**

MM, Mismatch; 0MM GvH, mismatched Allotype in GvH vector; 1MM GvH (A), 1 mismatched Allotype in GvH vector (rs9277534 A); 1MM GvH (G), 1 mismatched Allotype in GvH vector (rs9277534 G); 2MM, both DP alleles mismatched. Statistical significance marked in bold. Statistical trend is underlined.

mismatches significantly increased the risk of aGvHD, this didn't translate into higher mortality. This doesn't seem to be the case for relapse, where matched or permissively mismatched cases showed a markedly higher mortality related to relapse (42.6%) compared to non-permissively mismatched cases (33.8%), (data shown in Supplemental Material, **Table S4**). The additional HLA mismatch this time did not seem to impact the DP match effect as similar relapse-related death rates were observed for the DP compatibility groups analyzed in both, 10/10 and 9/10 HLA settings (data not shown). One additional factor is the time dependence of DP mismatch effect on GRFS, as from day 100 post HSCT it ceased to be significant (27).

Multivariate Models were checked for interaction between HLA-DPB1 and other classical HLA-mismatches by forming an interaction term, which showed not statistical significance. This is also shown in the separate analysis of 10/10 HLA and 9/10 HLA matched cases, where the respective effect of HLA-DPB1 mismatch did not appear to be influenced by the prevalence of an additional HLA mismatch with the exception of relapse as already mentioned previously. This implies that HLA-DP mismatches confer their effect on outcome rather independently from additional HLA-mismatches. As far as mismatch directionality is concerned, our analysis revealed that non-permissive mismatches in GvH direction mainly drove the overall effect of higher aGvHD but also lower relapse risk when compared to non-permissive mismatches in HvG direction. The fact that no such effect was observed in NRM suggests that DPB1-mismatch-induced morbidity is not only restricted to aGvHD but may also affect other pathophysiological pathways such as conditioning associated toxicity or infections early after hematopoietic stem cell transplantation (29). A mechanism of interaction may be the upregulation of HLA-class II molecules during viral infection possibly aggravating the impact of DPB1 mismatches in such cases (30). It's also possible that this effect may be influenced by ATG/Campath treatment as well as post-transplant immunosuppression. This is supported by the fact that a similar effect was also seen in a cohort from the MD Anderson Cancer center (31) but not in a multicenter cohort of patients where the transplant was facilitated by the NMDP (27). As to the mismatch directionality effect on relapse, it could be immunobiologically underpinned by the notion that highly immunogenic patient mismatched HLA-DPB1 probably stimulates donor T-cells resulting in a better GvL effect. Similar observations have also been reported elsewhere (28, 32). Interestingly, this effect was not evident in the advanced-disease-stage patient group. This might be attributed, however, to weakened statistical power on account of multiple combinations.

In our analysis we explored the impact of HLA-DPB1 mismatches on GRFS, a composite endpoint now increasingly used for assessing the success of HSCT, as it simultaneously measures the proportion of patients free from disease and GvHD (33). We considered two degree levels for aGvHD, II-IV and III-IV. No marked differences were observed between the two subanalyses. As GRFS is a combined endpoint summarizing three events (occurrences of aGvHD, relapse or

death), different effects are measured together. Perhaps the most interesting finding of this analysis was the absence of vector effect, although the latter was evident in aGvHD and relapse. An explanation for that could be the opposite effect of GvH directed non-permissive DP mismatches on these two endpoints resulting in an overall dampened and statistically insignificant effect.

Aim of this study was to conjointly assess different DP mismatch alloreactivity predictive models so that an extended predictive model can be proposed. To this end we included in our analysis, along with the TCE3 algorithm, the HLA-DPB1 expression model as proposed by Petersdorf et al. (14). Assignment of the rs9277534-G polymorphism was done by inference based on linkage disequilibrium data. A recent study showed that such an approach could be highly accurate (20). The G allele expression within the mismatched recipient allotype was associated with higher incidence of aGvHD suggesting a dose effect of the mismatched HLA-allotype (15). Such an association has also been reported for HLA-C differences (34). A shortcoming of the HLA-DPB1 expression model approach by Petersdorf et al. is that it was only applied to single mismatched HLA-DPB1 cases with no data available as to the effect of double mismatched HLA-DPB1 cases in GvH direction, which do however occur with a frequency of around 23%. In the combined DP mismatch model we aimed at combining the TCE3 immunogenicity- with the HLA-DPB1 expression-model taking also into consideration the mismatch directionality as well as the overall number of DP mismatches with GvH vector. This way we formed a hierarchy out of all implicated factors. The most important observation from this combined analysis consists in that HLA-DP non-permissive mismatch effect appears to be aggravated by the prevalence of two overall DP mismatches in the GvH direction as well as by an anticipated higher expressed patient mismatched allotype. The impact of two DP mismatches in GvH direction on non-permissive mismatch effect appears to be stronger as it significantly enhances the effect on GRFS, NRM, aGvHD and relapse. This observation is clinically relevant considering that about 16% of HSCTs are expected to have a non-permissive HLA-DP mismatch with two overall DP mismatches in GvH direction. The expected increased surface expression of the patient non-permissively mismatched allotype, on the other hand, appears to be significantly evident only for aGvHD and relapse. In summary these findings suggest that the combination of non-permissive DP mismatches with 2 DP-allele-mismatches as well as of non-permissive mismatches with a highly expressed mismatched patient allotype should be avoided. A recent study of Petersdorf et al. suggested that the overall number of mismatches is mainly relevant in HLA-mismatched transplantations whereas the expression level of the mismatched allotype is important in fully HLA-matched cases (35). Due to smaller cohort size and therefore compromised statistical power, we have not been able to confirm these findings in our study, as 10/10 and 9/10 HLA matched cases were assessed together in our combined DP-mismatch model. All other combinations including non-permissive mismatches with no mismatch in the GvH direction or a single low expressed mismatched allotype seem to be tolerable. This analysis is not yet conclusive as to whether double permissive DP-allele-mismatches in GvH direction should also be avoided or not, although a clear trend was also seen in this group. It is of note, however that this subgroup corresponded to only 6.7% of all included cases. Our study results indicate that although the immunogenicity model and the expression model confer distinctive effects on outcome due to different underlying mechanisms, they may be combined for refined donor selection strategies. Nevertheless, due to the many different possible combinations more data are needed and larger studies are warranted before final conclusions are drawn.

Limitations of our analysis are the small sample size in some sub-analyses particularly in the combined DP mismatch model. Missing data has also been a substantial problem for CMV status and blood group as well as for date of development of acute and chronic GvHD in the EBMT promise registry database although in direct collaboration with the transplant centers we were able to collect a substantial proportion of these missing data. Still missing data in the final analysis showed a completely random pattern, indicating no data collection bias. Furthermore, our cohort represents patients transplanted in Germany and shows a large proportion of patients treated with ATG as part of the conditioning treatment as well as a low proportion of patients treated with mTOR inhibitor based immunosuppression, which may limit comparability with other cohorts showing different features.

In conclusion, our study confirms the previously reported detrimental effect of non-permissive HLA-DPB1 mismatches according to the TCE3 model in a large cohort of patients having been treated with unrelated HSCT in German transplant centers between 2000 and 2014. This effect was similarly present in 10/10 and 9/10 HLA- matched transplantations. The results of our combined assessment of distinct DP mismatch alloreactivity models indicate that the effect mediated by rs9277534 may be independent from the immunogenicity model underlying the TCE3 model. Furthermore, an additional dose effect of mismatched HLA-DPB1 allotypes in GvH direction is implied, at least for aGvHD and relapse incidence. The aforementioned findings support an extension of the TCE3 model for refined donor selection avoiding the putatively detrimental combinations of non-permissive DP mismatches with overall 2 DP mismatches as well as with a high expressed mismatched patient allotype (rs9277534-G). Larger future studies are anticipated to offer a clearer insight into the multifaceted immunogenicity features of HLA-DPB1 mismatches addressed in this study.

DATA AVAILABILITY STATEMENT

The data analyzed in this study are subject to the following licenses/restrictions: If required, the data can be reanalyzed by other groups within our premises. Requests to access these datasets should be directed to joannis.mytilineos@zkrd.de.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee of the University of Ulm. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DF, DM, CT, JM, and HS are principal investigators. They designed the study, performed data analysis/interpretation, and wrote the manuscript. DM and CT as well as DF and JM contributed equally. MH, CM, SaF, CN, and CT contributed to the data analysis and in writing of the manuscript. CN, UP, DB, MG, ED, GW, NK, NM, HE, KE, SeF, JC, MK, MD, BH, SK, and MR contributed the patients, reviewed the data, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.614976/full#supplementary-material

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Combined Immunotherapy With Belatacept and BTLA Overexpression Attenuates Acute Rejection Following Kidney Transplantation

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Background: Costimulatory blockade provides new therapeutic opportunities for ensuring the long-term survival of kidney grafts. The adoption of the novel immunosuppressant Belatacept has been limited, partly due to concerns regarding higher rates and grades of acute rejection in clinical trials. In this study, we hypothesized that a combined therapy, Belatacept combined with BTLA overexpression, may effectively attenuate acute rejection after kidney transplantation.

Materials and Methods: The rat kidney transplantation model was used to investigate graft rejection in single and combined therapy. Graft function was analyzed by detecting serum creatinine. Pathological staining was used to observe histological changes in grafts. The expression of T cells was observed by immunohistochemistry and flow cytometry. *In vitro*, we constructed an antigen-stimulated immune response by mixed lymphocyte culture, treated with or without Belatacept and BTLA-overexpression adenovirus, to observe the proliferation of receptor cells and the expression of cytokines. In addition, western blot and qRT-PCR analyses were performed to evaluate the expression of CTLA-4 and BTLA at various time points during the immune response.

Results: In rat models, combined therapy reduced the serum creatinine levels and prolonged graft survival compared to single therapy and control groups. Mixed acute rejection was shown in the allogeneic group and inhibited by combination treatment. Belatacept reduced the production of DSA and the deposition of C4d in grafts. Belatacept combined with BTLA overexpression downregulated the secretion of IL-2 and IFN-γ, as well as increasing IL-4 and IL-10 expression. We also found that Belatacept combined with BTLA overexpression inhibited the proliferation of spleen lymphocytes. The duration of the elevated expression levels of CTLA-4 and BTLA differentially affected the immune response.

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Conclusion: Belatacept combined with BTLA overexpression attenuated acute rejection after kidney transplantation and prolonged kidney graft survival, which suggests a new approach for the optimization of early immunosuppression after kidney transplantation.

Keywords: kidney transplantation, Belatacept, acute rejection, BTLA, CTLA-4, immunosuppressant

INTRODUCTION

Compared to classic maintenance dialysis, kidney transplantation is considered to be an optimal treatment option with improvement in life quality and prolongation of survival in end-stage renal disease patients (1, 2). In recent years, along with the amelioration of surgical techniques, popularization of organization matching and application of new immunosuppressants, apparent improvement has been observed in short-term graft survival among recipients (3). Nevertheless, ensuring the long-term survival of kidneys after transplantation remains an important objective that involves the consideration of a number of immunological and nonimmunological factors, especially acute rejection (4, 5). Therefore, immunotherapy against acute rejection still attracts considerable attention in the clinic (6).

Acute rejection occurs most commonly within three months after transplantation and clinically results in decreased urine, increased serum creatinine, swelling and pain of graft (7). Based on pathology, there are two types of acute kidney allograft rejection: T-Cell Mediated Rejection (TCMR) and Antibody-Mediated Rejection (ABMR). TCMR is caused by the immune response between T cells and antigens present in recipient transplanted kidneys (8), with tubulitis, interstitial inflammation and especially intimal arteritis as a feature. In addition, antigen-specific binding of receptor circulating antibody to endothelial cell allografts leads to ABMR, which is characterized by the histologic evidence of acute tissue injury including glomerulitis and peritubular capillaritis as well as the existence of circulating donor specific antibody (DSA) and deposition of complement degradation products C4d in grafts (9). Immunosuppressive regimens for acute rejection therapy have been the focus of transplant research. However, increasing clinical concerns have occurred with nephrotoxicity and negative effects due to the excessive immunosuppression of current immunosuppressants such as calcineurin inhibitors (CNIs) and negative effects of overimmunosuppression (10, 11). Furthermore, effective treatments are still lacking in acute ABMR, correlated with severe clinical symptoms and poor prognosis (12). Consequently, there remains a need for further optimized therapeutic strategies to prevent allograft rejection (13).

Costimulatory and coinhibitory pathways, which are the second signals of T cell activation, play an essential role in transplantation immunity. Several research studies have indicated that coinhibitory molecules, such as PD-1 and CTLA-4, can transmit negative signals by attenuating T cell activation, inhibiting cell proliferation and inducing immune tolerance (14). Enhancing coinhibitory signals has potential clinical application value in transplantation immune-regulation and rejection inhibition (15). Belatacept (CTLA-4 fusion protein) suppresses T cell activation *via* competitively blocking the binding of the antigen-presenting cell (APC) surface molecules CD80\CD86 to CD28 and has been approved by the FDA against acute rejection (AR) in renal transplant recipients in 2011 (16). Retrospective studies reported a lower risk of hypertension and cardiovascular disease, similar graft survival, as well as sustained improvement in renal function following treatment with Belatacept when compared to CNI-treated patients (17–19). Additionally, Belatacept showed remarkable benefits in recipients with CNI intolerance or chronic allograft nephropathy (20). However, the high incidence of acute cellular rejection after surgery is one of its limitations, and the rate of AR in the Belatacept group was up to 24% at three years in the clinical BENEFIT trial. Infection risk, urinary tract infections and cytomegalovirus infections were most common, and adverse reactions of maintenance dose, such as posttransplant lymphoproliferative disorder, cannot be ignored (21, 22). In fact, the high-cost burden is another obstacle for Belatacept popularization. Therefore, the routine clinical application of Belatacept is controversial, especially in recipients diagnosed with AR.

A newly found coinhibitory molecule, BTLA is expressed widely in innate and adaptive immunocytes and increases expression when activated (23). The inhibitory effect of BTLA on the immune response has been confirmed by recent studies; for instance, targeted BTLA therapy can inhibit rejection in a mouse heart transplantation model (24) but less so in kidney transplantation. Our previous research has shown that the BTLA pathways were involved in the pathogenesis of AR in biopsy-proven recipients following kidney transplantation, and BTLA overexpression can suppress TCMR by regulating T cell receptor downstream signals (25, 26). Additionally, several studies indicated the potential value of combining costimulatory or costimulatory molecules in disease treatment, which may reduce adverse effects through a lower single dose and provides new ideas for the prevention of kidney transplant rejection (27). Based on these findings, we speculated that Belatacept combined with the BTLA pathway can ameliorate the occurrence of acute rejection following kidney transplantation, inhibit T cell activation and proliferation in recipients, improve kidney graft functions and prolong graft survival. This study investigated this hypothesis by using a rat renal transplant model of acute rejection and mixed lymphocyte reaction *in vitro* experiments.

MATERIALS AND METHODS

Ethics Statement

All animal studies were strictly performed following the Nanjing Medical University Animal Care and Use Committee guideline (Ethical Approval Number: IACUC1601140-1).

Animals and Reagents

Major histocompatibility complex (MHC) fully mismatched SD and Wistar rats were purchased from Charles River Laboratory (Beijing, China). Belatacept was obtained from Bristol-Myers Squibb (NY, USA), and BTLA overexpression adenovirus and negative-control vectors (CMV-MCS-3FLAG-SV40-EGFP, which is a linear double-stranded DNA virus with a wide host range and the ability to infect dividing and non-dividing cells) were constructed by Genechem (Shanghai, China). Anti-CTLA-4 (Santa-Cruz, USA), anti-BTLA (Abbiotec, USA), anti-GAPDH (Abcam, USA), anti-CD3 (Abcam, USA) and anti-Foxp3 (Abcam, USA) antibodies were used for Western blot or immunohistochemistry (IHC) staining. Anti-C4d (American Research Products, USA) and anti-CD138 (Abcam, USA) antibodies were obtained for immunofluorescence staining. We obtained the flow antibodies APC-labeled anti-CD3, FITC-labeled anti-CD4 and PerCP-eFluor710-labeled anti-CD8 from eBioscience (CA, USA). We used rat GM-CSF, IL-4 and TNF-α (Prospec-Tany, ISR) to stimulate dendritic cells (DC).

Kidney Transplantation Model

Rat kidney transplantations were carried out according to a previously described procedure (26). Two experienced microsurgeons performed the surgeries in a sterile environment. In brief, we separated and removed the donor rat left kidney and ureter and then transplanted it to the left renal fossa of recipients that underwent bilateral nephrectomy. The renal arteriovenous was anastomosed end-to-end, and the ureter was embedded into the recipient's bladder. The renal artery was seen to pulsate, the ureter engorged, and the graft returned to a ruddy complexion. The whole surgical procedure was completed in 2 h with an anastomotic time of approximately 40 min. The surgery-related data for each group are shown in **Table S1**.

Rats were randomly divided into different treatment groups (n=5 for each group and time-point). Kidneys from SD rats transplanted into SD rats constituted the Syngeneic (Syn) group. Wistar rats were donors with the SD rats as recipients in the Allogeneic (Allo) group to induce acute rejection. The other groups consisted of (1) the Allo+ Control group: the allogeneic recipients were pretreated negative-control vectors two days before surgery; (2) the Allo+BEL group: Belatacept (60 mg/kg) was injected into SD rat abdomens at postoperative and 4 days after transplantation; (3) for the Allo+ BTLA-Over group, the SD recipients were preinjected with BTLA overexpression adenovirus (1×109 PFU/each) two days posttransplant; and (4) for the Allo+Combination group, the recipients were pretreated with BTLA adenovirus and administered Belatacept (**Figure S1**). The recipients were harvested for graft tissue and blood at each time point. Additionally, eight recipients in each group were observed for graft survival. We observed postoperatively the urine output of recipients, and anuria was considered the end of kidney graft survival (28).

Histopathology Examination

The detailed procedure has been described previously (26). The harvested grafts were placed into 10% buffered formalin and then stored in paraffin. These tissues embedded were sectioned at a 4 μm thickness. Hematoxylin and eosin (HE) staining was done in accordance with standard techniques. Pathological manifestations of acute rejection were evaluated based on Banff 2017 classification (29)

Immunohistochemistry and Immunofluorescence Staining

Immunohistochemistry was performed to detect the expression of CD3, CTLA-4 and BTLA in graft and Foxp3 in the spleen of recipients. The operation method refers to the standard protocol. Isotype control using a non-immune antibody of the same isotype and at the same concentration as the primary antibody was performed as staining control. Then, we took 8 high-resolution images under the microscope and used image pro plus5.0 software to measure the relative expression of the target protein with integrated optical density (IOD). We used the graft tissue sections with anti-C4d (dilution: 1:50) and anti-CD138 (dilution: 1:500) antibodies and corresponding secondary antibodies to do immunofluorescence staining. The fluorescence intensity per unit area was used to analyze the relative expression in the kidney.

Serum Creatinine Detection

To analyze the change in renal function of the recipient rats, we used a creatinine detection kit (Jiancheng BI, China) to detect the serum creatinine level according to the manufacturer's instructions.

Mixed Lymphocyte Reaction

In this study, primary SD rat spleen lymphocytes were used as immune responder cells in a mixed lymphocyte reaction. We extracted dendritic cells from the peripheral blood of Wistar rats and then cultured them in complete medium (containing 1640 medium, FBS, 50 ng/ml Rat-GM-CSF, 10 ng/ml Rat-IL-10 and 20 ng/ml Rat-TNF-α) for 7 days, which allowed them to become mature dendritic cells (mDCs) and act as antigen-presenting cells. The mDCs were counted and then evenly spread in 12-well plates with 5×10^4/well or in 96-well plates (2×10^5/well). We pretreated these mDCs with mitomycin C (30 mg/ml) in serum-free medium for 20 min before admixture. As response cells, the extracted primary spleen lymphocytes were laid in 12-well plates (1×10^6/well) and 96-well plates (2.5×10^5/well) to form mixed lymphocyte reactions. Cultures were maintained in complete medium for the required times at 37°C in 5% CO2 in the air. The reaction system and other details about treatment are shown in the results section.

Western Blot Analysis

The graft tissues and response cells of MLR were collected and lysed in RIPA buffer with protease and phosphatase inhibitors. We extracted the proteins of tissues and cells following previous work and then determined protein concentrations. Equal quality proteins underwent polyacrylamide gel electrophoresis, were separated on 10% SDS gels and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking and washing, these membranes were incubated with anti-CTLA-4, anti-BTLA and anti-GAPDH antibodies. The band intensity and volume were clarified to examine the relative expression. All experiments were repeated three times.

Quantitative Reverse Transcription-PCR

Kidney grafts were submerged in RNAlater stabilization solution (Sigma, USA) for freezing. Total RNA of graft tissues and cells was extracted using RNA extraction kits (Tiangen, China) and reverse transcribed to cDNA by a PrimeScript RT Kit (Takara, Japan). Quantitative RT-PCR was carried out *via* the SYBR Green PCR kit (Takara, Japan) according to the manufacturer's instructions. The 2-ΔΔCt method was adopted to analyze gene expression. The sequences used in our study are as follows:

CTLA-4: forward: 5'-AGTGACCCAACCTTCAGTGG-3',
reverse: 5'-AAGCCCAACGTGTTCTTCAC-3';
BTLA: forward: 5'-ATCCCAGATGCTACCAATGC-3',
reverse: 5'-TTGGGAGTTTGTCCTGGAAC-3';
GAPDH: forward: 5'-GGCCTTCCGTGTTCCTACC-3',
reverse: 5'-CGCCTGCTTCACCACCTTC-3'. All experiments were repeated three times.

Enzyme-Linked Immunosorbent Assay

We tested serum samples and supernatants of MLR by ELISA according to the instructions of the kits (Cusabio, China). At 450 nm, the OD values of each sample were measured to express the concentration of cytokines.

Donor Specific Antibody Detection

We harvested spleens from donor Wistar and recipient SD rats as probes to detect DSA in serum. The fresh spleen cells were mashed through 70 μm filters, resuspended in PBS, and then added into 96-well plates with 5×10^5/well after washed twice. The serum from kidney transplanted rats was diluted (1:50) and used to incubate the spleen cells for 30 min at room temperature. The cells were then washed and incubated with FITC-labeled anti-rat-IgG antibody (Jackson ImmunoResearch, PA, USA) for 30 min. DSA was measured by assessing anti-IgG signal by flow cytometry, with the incubated spleen cells from SD rats as staining control and expressed as the mean fluorescence intensity (MFI).

Flow Cytometry

To explore the cell proliferation response in MLR, we used bromodeoxyuridine (BrdU) incorporation and then tested by flow cytometry. The protocol followed previous research. Additionally, peripheral blood of rat recipients at 7 days after transplantation was harvested and stained with APC-labeled anti-CD3, FITC-labeled anti-CD4 and PerCP-eFluor710-labeled anti-CD8 antibodies. Flow cytometry was determined by a Gallios flow cytometer and analyzed with FlowJo Software (Tree Star, OR).

Statistical Analysis

Student's t-test was used to compare the difference in expression levels among groups, and all data are expressed as the mean ± standard deviations (SD). To compare the differences in graft survival, we performed Kaplan-Meier survival analysis and a log-rank test to compare the graft survival among each group. P-values of less 0.05 were considered to be significant. GraphPad Prism, version 8.0 (GraphPad Software) was used for statistical analysis.

RESULTS

Establishment and Identification of Rat Renal Transplantation Model With Acute Rejection

To investigate the role and mechanism of the combination therapy on acute rejection *in vivo*, we established the acute rejection model of orthotopic kidney transplantation in rats with 5 recipients per timepoint in each group. The grafts were harvested for pathology analysis preoperatively and postoperative days 1, 3, 5 and 7. **Figure 1A** indicates that, in contrast to the preoperative state, the Syn group showed ischemia-reperfusion injury, renal tubular edema, and other mild acute renal injury caused by surgery on days 1 to 7, without acute rejection reactions. However, in grafts from the Allo group, we observed acute renal injury at day 1; subsequently, further manifestations with mononuclear cell infiltration, glomerulitis, and tubular injury could be seen from day 3 to day 5, which progressed to pathological mixed acute rejection characterized by moderate to severe intimal arteritis, glomerulitis, and peritubular capillaritis at day 7. Further statistical analysis based on Banff 2017 revealed a significant increase in all classification scores after allogeneic transplantation (**Figure 1B**). IF staining showed that linear C4d sedimentation in peritubular capillaries was increased in the Allo group compared to the Syn group. Serum donor specific antibody IgG was remarkably upregulated in the Allo group. (**Figures 1C–E**) Similarly, the Allo+Control group pretransfected by negative vector also exhibited progressive aggravating AR from day 3 to day 7, clear C4d sedimentation and IgG positivity at day 7 (**Figure S2**). Overall, classic pathological evidence of cell- and antibody-mediated rejections was present in the allogeneic group and was most pronounced at postoperative day 7, and there was no significant effect of negative vector intervention in the Allo+Control group.

Combination Therapy Improved Renal Function and Prolonged Graft Survival

First, we confirmed the effectiveness of intravenous overexpression adenovirus in normal SD rats by IHC, western blot and qRT-PCR analyses (**Figure S3**). Then, serum samples were collected for creatinine testing after transplantation to reflect renal function changes. We found that the Scr in the Allo group was consistently increased after surgery, which was significantly different than the Syn group. Belatacept, BTLA overexpression and the combination therapy can inhibit creatinine increase after kidney transplantation compared to the Allo+Control group (**Figure 2A**). Notably, although there was no significant difference between the Allo+Combination group and the Allo+BTLA-Over group, the combined intervention resulted in lower Scr values with a decreasing trend.

Eight recipients per group were constructed to evaluate graft survival. In contrast to the long-term survival in the Syn group, the median survival time was 7 days in the Allo group, which was also similar to the Allo+Control group. Surprisingly, the combination therapy clearly prolonged graft survival to 17 days, which was superior to the survival obtained with single

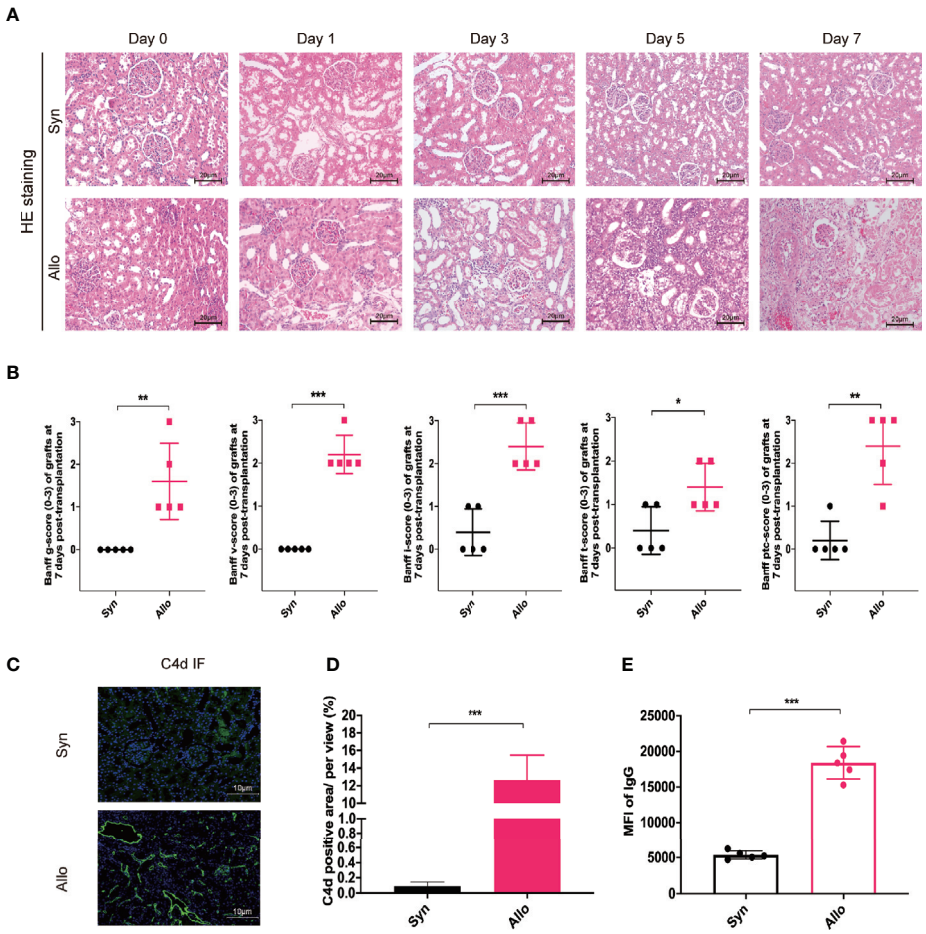

FIGURE 1 | Construction of rat renal transplantation model with acute rejection. **(A)** Pathological staining analysis of kidney grafts from the Syngeneic group and the Allogeneic group recipients on preoperative Day 0 and postoperative Days 1, 3, 5 and 7 (Magnification: 200×). **(B)** Assessment of graft tissues glomerulitis (g), intimal arteritis (v), interstitial inflammation (i), tubulitis (t), and peritubular capillaritis (PTC) based on the Banff 2017 classification system. **(C)** Graft tissue-specific C4d immunofluorescence staining analysis on 7 days after transplantation (Magnification: 400×). **(D)** The proportion of C4d-positive regions was used to compare relative C4d-positive expression across groups. **(E)** Expression of serum DSA in each group were reflected by mean fluorescence intensity (MFI) of donor-related IgG in flow cytometry analysis. Results are expressed as mean ± SD, NS, no significant; *P < 0.05; **P < 0.01; ***P < 0.001.

therapy (**Figure 2B**). These results show that combining Belatacept and BTLA overexpression attenuated creatinine elevation induced by acute rejection, improved postoperative graft renal function and significantly prolonged graft survival.

Combination Therapy Effectively Suppressed Acute Rejection After Kidney Transplantation

To investigate the effect of single intervention versus combination therapy on acute rejection, we performed characteristic pathological staining and DSA detection on recipient specimens from each group at postoperative 7 days (**Figure 2C**). What stands out in this figure is the clear decrease of glomerulitis and peritubular capillaritis in the Allo+BEL group compared with the Allo+Control group, while mild to moderate intimal arteritis was rarely seen under 400× microscopy. Only mild interstitial inflammation, tubulitis, and glomerular edema were shown in the BTLA overexpressed and combination treatment group without apparent cell-mediated rejection, such as intimal arteritis in the high-fold field.

Positive detection of C4d in peritubular capillaries and DSA in serum are features of ABMR. Further studies reported that C4d in graft IF staining were notably reduced in the Belatacept treatment group, as well as in the Allo+Combination group, compared to the Allo+Control group (**Figures 2C, D**). In addition, flow cytometry analysis revealed that serum DSA was particularly inhibited in the Allo+Combination group, rather than the Allo+Control group, which was caused by the reduction of antibody-producing CD138-positive plasma cells in grafts with combination therapy (**Figures 2C–F**). An additional interesting result that emerged was a more effective downregulation of CD138 infiltration and DSA production with Belatacept than single BTLA overexpression treatment. These findings indicate that Belatacept has not only a limited effect on TCMR but also a

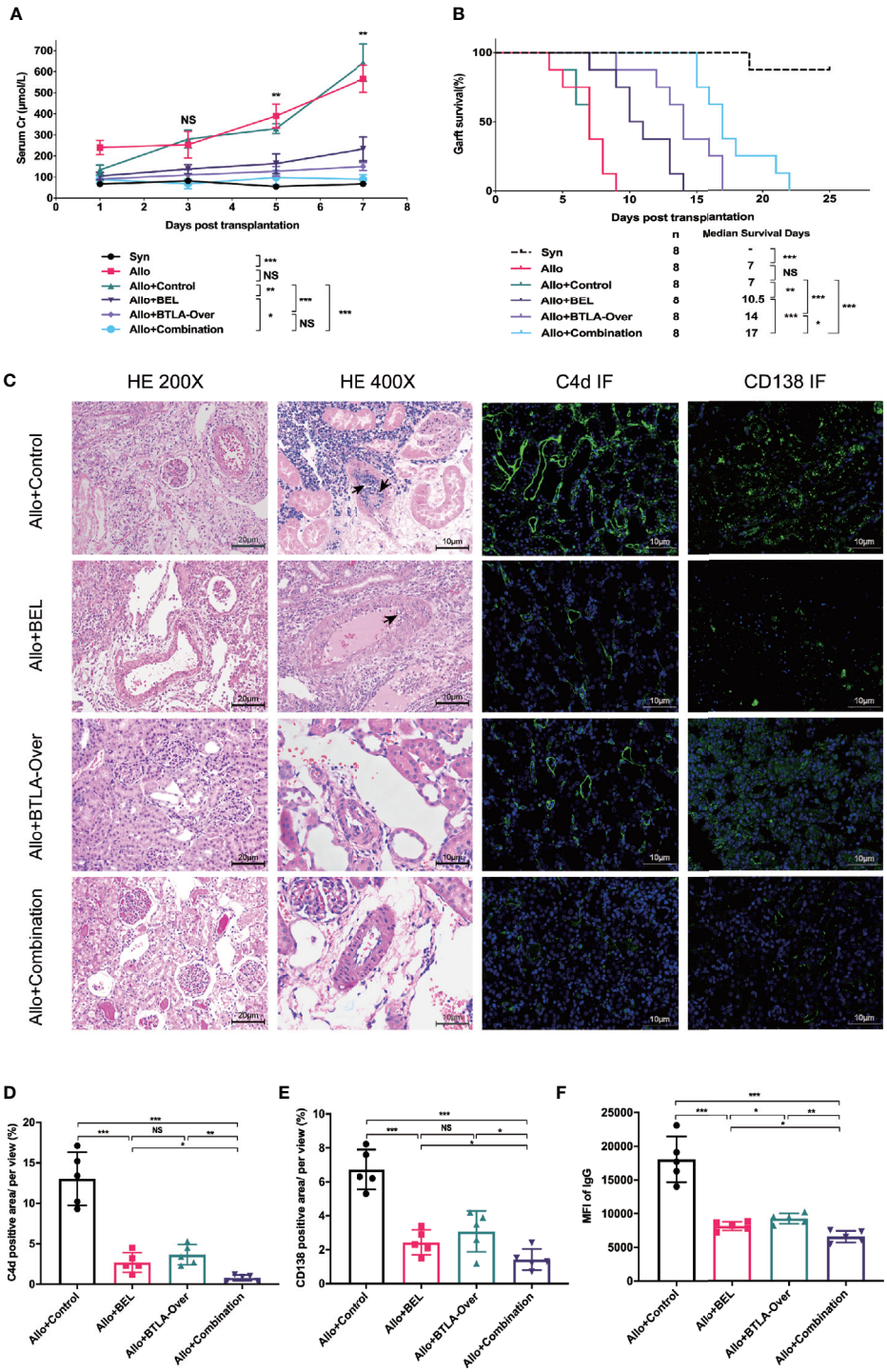

FIGURE 2 | Combination therapy improved renal function, prolonged graft survival and effectively suppressed acute rejection after kidney transplantation. **(A)** Serum creatinine levels at various timepoints after kidney transplantation among each group of recipient rats. **(B)** Analysis of graft survival time posttransplantation of recipient rats. **(C)** Postoperative day 7, recipient kidney graft tissue-specific HE and immunofluorescence staining analysis. Magnification: HE for nephron and renal interstitium: 200×; HE for arterial intima: 400×, Arrows: intimal arteritis; C4d immunofluorescence staining: 400×; CD138 immunofluorescent staining, 400×. **(D)** The proportion of C4d-positive regions was used to compare relative C4d-positive expression across groups. **(E)** Percentage of CD138-positive regions used to express relative CD138 expression levels in tissues. **(F)** MFI of IgG in flow cytometry was used to reveal the expression of serum donor-specific antibody (DSA) in each group. NS, no significant; *P < 0.05; **P < 0.01; ***P < 0.001.

significant inhibitory effect on ABMR; overexpression of BTLA can obviously attenuate TCMR characterized by arterial endarteritis, and combination treatment can significantly inhibit mixed acute rejection.

Combined Belatacept and BTLA Overexpression Affected T Cell Frequency in Recipients

Both Belatacept and BTLA target the T cell surface coinhibitory molecules, which mainly affect the proliferation and activation of T cells. Therefore, we further observed T cell changes to explore the specific mechanism. Data from IHC staining indicated that the Allo+Control group had more CD3 infiltration in graft tissues, whereas both Belatacept and BTLA overexpressed reduced CD3 expression (**Figures 3A, B**). This result suggests that combination therapy suppressed T cell infiltration in the graft during the acute rejection period. To research the source of the reduction of T cell infiltration, we analyzed CD3, CD4 and CD8 positive cells in peripheral blood by flow cytometry (**Figure 3C**). We found that combined Belatacept and BTLA overexpression significantly reduced the composition of CD3+ T cells in total lymphocytes in the postoperative peripheral blood compared with the control group (**Figure 3D**).

At the same time, we observed the expression of CD4+ and CD8+ cells in CD3+ T cells. The inhibitory effect of targeted BTLA on CD3+CD4+T cells was stronger than that of Belatacept treatment alone and largely consistent with the combination treatment (**Figure 3E**). Notably, there was no significant difference in CD3+CD8+ T cell expression between these groups (**Figure 3F**). Taken together, these results indicate that the combined treatment mainly inhibited CD4+ T cells, rather than CD8+ T cells, in recipient peripheral blood lymphocytes after kidney transplantation to reduce the total number of CD3+ T cells and subsequently attenuate T cell infiltration in the grafts. Meanwhile, the inhibitory effect of BTLA overexpression on T cells was stronger than that of Belatacept.

Belatacept Combined With BTLA Overexpression Inhibited T Lymphocyte Proliferation

We conducted MLR to stimulate antigen-specific immune response *in vitro* to initially test the effects of Belatacept and BTLA on cell proliferation. The BrdU positive rate in response cells was detected by flow cytometry at 3 days of MLR to reflect the change of proliferation (**Figure 4A**). The results, as shown in **Figure 4B**, suggest that compared with normal splenocytes (the Naïve group), the cell proliferation rate of the MLR group was significantly higher after stimulation. Furthermore, Belatacept inhibited T lymphocyte proliferation in a dose-dependent manner.

Western blot and qRT-PCR analyses were used to verify the transfection efficiency of BTLA adenovirus (**Figure S4**). Then, recipient cells were pretransfected with BTLA-overexpression adenovirus 2 days prior to MLR and intervened with 10 ug/ml Belatacept in the combined treatment group (**Figure 4C**). No significant difference in cell proliferation was found between the negative vectors in the Control group and the MLR group. Compared to the Control group, lymphocyte proliferation was significantly inhibited by Belatacept combined with BTLA overexpression (the BEL+BTLA-Over group) (**Figure 4D**). These results suggest that Belatacept, overexpression of BTLA, and combination therapy inhibited lymphocyte proliferation *in vitro*.

Belatacept and BTLA Overexpression Combination Affected Cytokine Production

ELISA was performed to examine the expression of cytokines IL-2, IFN-γ. IL-4 and IL-10. In an *in vivo* experiment, the results showed that serum IL-2 and IFN-γ expression were significantly decreased in the Allo+Combination group compared to the Allo+Control group. In addition, Belatacept treatment upregulated IL-4 and IL-10 levels compared with negative control, more significantly than BTLA overexpression, and combination treatment similarly stimulated IL-4 and IL-10 secretion. These results indicate that combined therapy inhibited the secretion of serum IL-2 and IFN-γ, as well as induced the production of IL-4 and IL-10 (**Figures 5A–D**). According to Foxp3 IHC analysis of postoperative spleen tissues, we found that Belatacept combined with BTLA overexpressed significantly upregulated Foxp3 expression in spleen tissues compared to the control group (**Figures 5E, F**).

Subsequent analysis was done in MLR to examine the expression of cytokines in the supernatant after 72 h *in vitro* (**Figures 5G–J**). The Control group pretransfected with adenovirus negative vectors did not differ significantly from the normal MLR group in terms of individual cytokine expression. Similar to the *in vivo* results, the cointervention of BEL+BTLA-Over significantly inhibited the secretion of IL-2 and IFN-γ in response cells but upregulated the expression levels of IL-4 and IL-10 in the supernatant compared with the Control group ($P<0.05$). Interestingly, there was no significant difference of IFN-γ in the BEL group compared with the Control group, and Belatacept stimulated the secretion of IL-4 and IL-10. The above experimental results show that Belatacept and BTLA overexpression changed cytokine expression levels, suggesting possible changes in the differentiation of T cells secreting these cytokines.

CTLA-4 and BTLA Expression Upregulation in Antigen-Specific Immune Responses Differed Over Time

To explore the differences in the effects of Belatacept and overexpressed BTLA along with the mechanisms of combined treatment on cell differentiation, we evaluated the expression of CTLA-4 and BTLA at different times in an *in vivo* model and in an *in vitro* model. IHC staining of grafts showed that CTLA-4 expression levels on postoperative days 1 to 3 were similar to those of the preoperative period; however, CTLA-4 infiltration increased in tissues on postoperative day 5, increasing more than 40-fold compared with preoperative day 0. BTLA expression was rapidly increased on day 1, then began to decrease on day 3, and by day 7 expression levels were lower than day 1 (**Figures 6A–C**). In addition, we extracted transplanted kidney protein and RNA

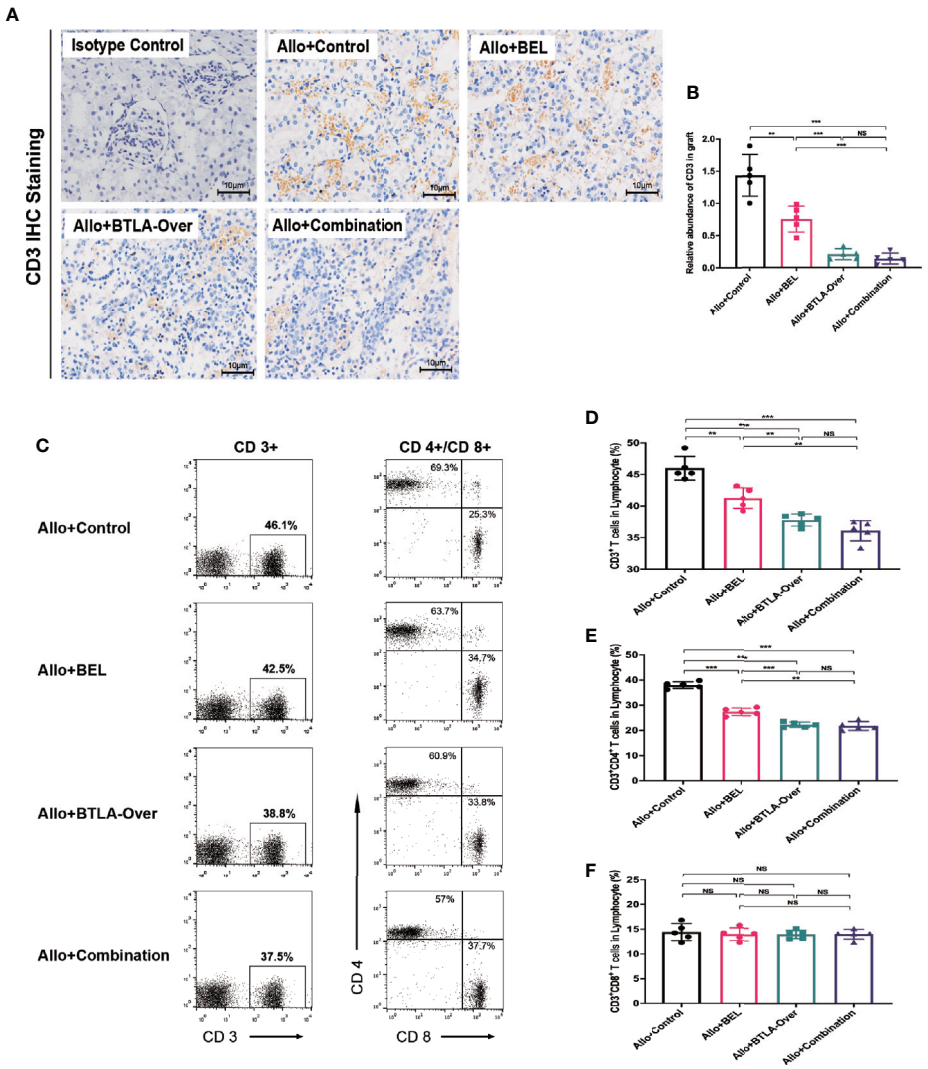

FIGURE 3 | Combination therapy inhibited receptor peripheral T cells and reduced graft CD3+ cell infiltration. **(A)** Analysis of CD3+ cell infiltration in transplanted kidney tissue by immunohistochemical staining. Magnification: 400×. **(B)** Integral optical density value (IOD) was used to indicate the relative expression of CD3 in the tissue. **(C)** Flow cytometry to detect CD3, CD4, CD8 positive cell frequency in peripheral blood. CD4+/CD8+ cells were circled from CD3+ cells in total lymphocyte. **(D)** Percentage of CD3+ cells in total lymphocytes. **(E)** Percentage of CD3+CD4+ cells in each group to total lymphocytes. **(F)** Percentage of CD3+CD8+ cells to total lymphocytes in each group. Results are expressed as mean ± SD, NS, no significant; **P < 0.01; ***P < 0.001.

and measured CTLA-4 and BTLA expression by western blot and qRT-PCR analyses, which matched the above results (**Figures 6D–H**). These findings indicate that both CTLA-4 and BTLA were upregulated in the early stages of acute rejection, and BTLA expression was increased earlier than CTLA-4.

Then, we examined the protein and mRNA expression of CTLA-4 and BTLA in receptor cells at 0, 6, 12, 24, 48, and 72 h after MLR to observe the time-trend (**Figures 6I–M**). We found that CTLA-4 upregulated mRNA expression at 24 h after stimulation and showed high protein expression at 48 h, while BTLA increased mRNA expression at 6 h and then began to decrease as well as upregulated BTLA protein at 12 h. These trends are similar to those of *in vivo* experiments and suggest that CTLA-4 and BTLA may act early in the immune response after antigenic stimulation, and BTLA expression was elevated earlier than CTLA-4.

DISCUSSION

Organ transplantation is the most effective treatment for organ failure, especially for the kidney (30). Nevertheless, the occurrence of acute rejection posttransplantation as a major reason for allogeneic graft dysfunction affects long-term survival, which calls for further exploration and optimization of immunosuppression treatment. Combined targeting of coinhibitory molecules provides a novel approach for the

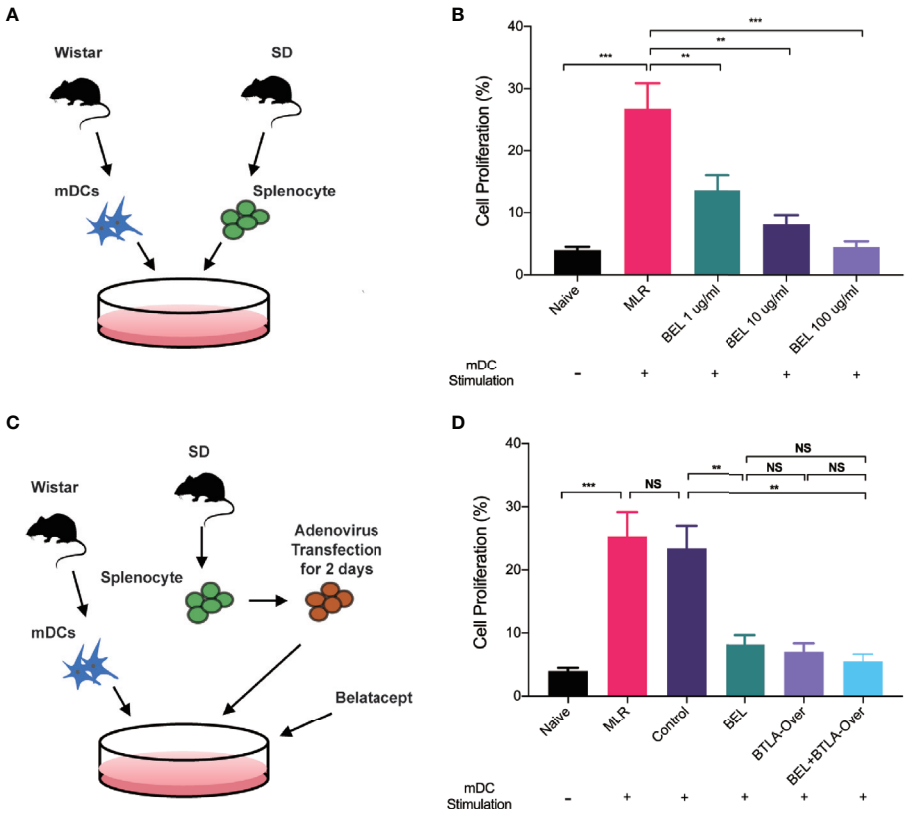

FIGURE 4 | Belatacept combined with BTLA overexpression inhibited lymphocyte proliferation. **(A)** Schematic of the mixed lymphocyte reaction, extracting mature dendritic cells from Wistar rat as stimulus cells and SD rat spleen lymphocytes as receptor cells for mixed culture. **(B)** Proliferative changes in lymphocytes after different doses of Belatacept intervention by BrdU incorporation and flow cytometry analysis. **(C)** Schematic diagram of combination treatment in mixed lymphocyte cultures. **(D)** The proliferation of receptor lymphocytes under single versus combination intervention. Results are expressed as mean ± SD, NS: no significant; **P < 0.01; ***P<0.001.

prevention and treatment of acute rejection. In the present study, we found that Belatacept combined with BTLA altered T cell differentiation, reduced DSA production, inhibited mixed rejection as well as prolonged graft survival.

We initiated antigen-specific immune responses *in vitro* through MLR and observed that both single and combination therapy effectively inhibited receptor cell proliferation. This finding suggests that Belatacept linked with BTLA overexpression reduced the degree of the alloimmune response and potentially functions to inhibit rejection after kidney transplantation. As previously described (31), we selected MHC fully mismatched Wistar and SD rats to construct a homozygous allogeneic kidney transplantation model to induce the onset of acute rejection *in vivo*. The allogeneic rats underwent a significant increase in postoperative serum creatinine, a dramatic loss of graft function and anuria around day 7. Allogeneic grafts showed worse pathological changes such as interstitial inflammation and tubulitis after transplantation, and by the seventh day, there was severe intimal arteritis and peritubular capillaritis, which revealed the coexistence of acute cell- and antibody-mediated rejection. Several studies have approved a correlation between C4d-positive staining, DSA, and histopathological findings in ABMR (32, 33), and the deposition of pericapillary complement C4d is highly suggestive of severe humoral rejection responses (34, 35). In this model, allogeneic recipients showed significantly elevated DSA on postoperative day 7, accompanied by C4d-positive staining with deposition of CD3-positive T cells in the transplanted kidney, which is similar to previous findings (36). These findings all point to the existence of severe mixed acute rejection.

As a maintenance drug, continued Belatacept administration contributes to the prevention and treatment of acute rejection and maintains long-term graft survival (37). In this study, we administered Belatacept twice postoperatively in rats, and the results showed that early noncontinuous administration failed to induce immune tolerance. Considering that adenovirus achieves stable expression 1–2 days after injection and can maintain its effect for approximately 7–10 days, we injected it 2 days preoperatively, resulting in BTLA overexpression. Although the single adenovirus treatment also failed to produce immune tolerance, the graft median survival time was prolonged to 14 days, which is similar to other findings of BTLA in organ transplantation (26, 38). As we surmised, after combination therapy, the recipients showed remarkable

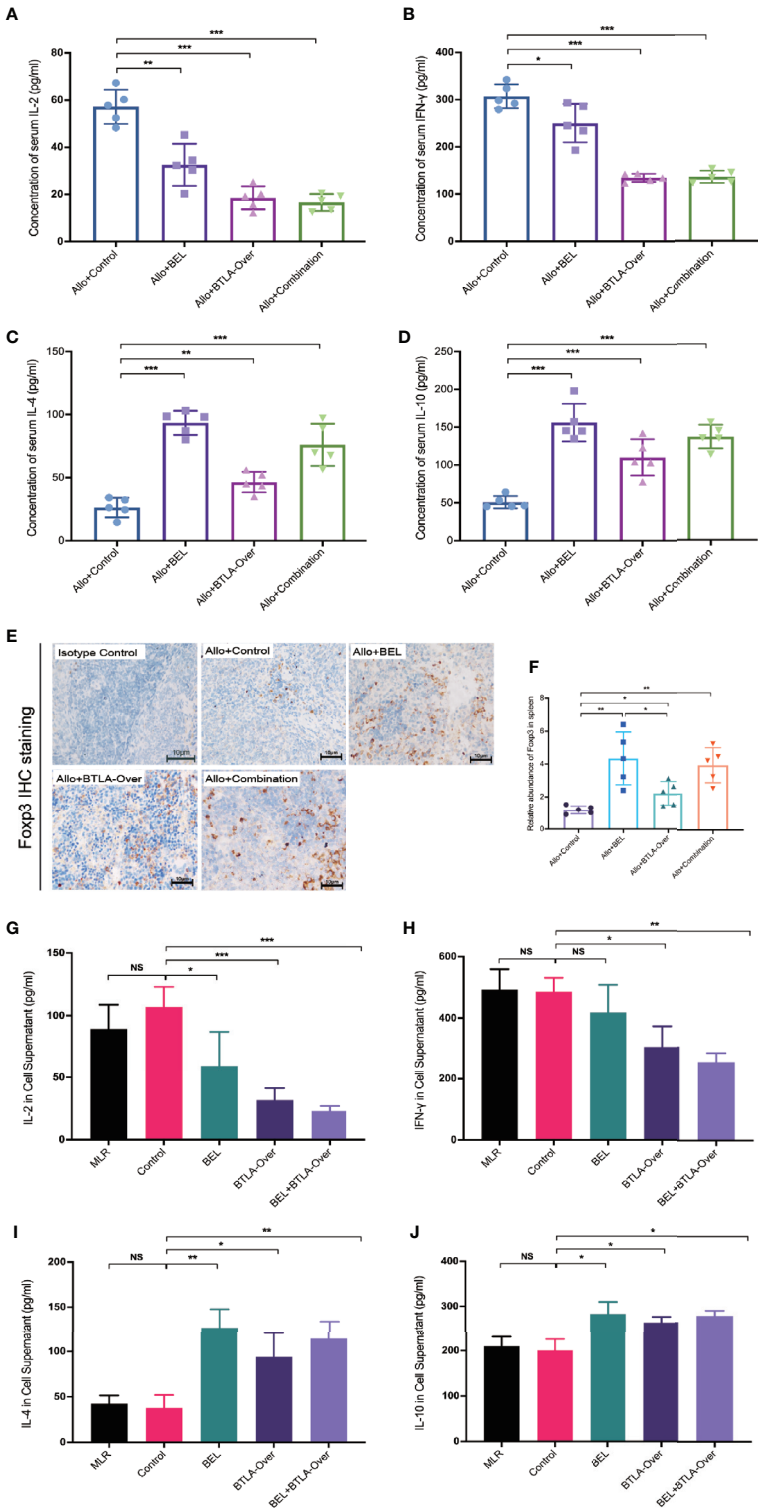

FIGURE 5 | Belatacept combined with BTLA overexpression affected T cell differentiation in immune response. **(A–D)** ELISA test for serum cytokine expression levels in recipient rats on day 7 after kidney transplantation. (**A**: IL-2; B: IFN-γ; C: IL-4; D: IL-10). **(E)** Immunohistochemical staining analysis of Foxp3 in recipient spleen tissues on day 7 after surgery. Magnification: 400×. **(F)** Integral optical density value (IOD) was used to indicate the relative expression of Foxp3 in spleen tissue. **(G–J)** ELISA was used to detect cytokine expression in supernatants of mixed lymphocytes after 72 h of culture. (**G**: IL-2; H: IFN-γ; I: IL-4; J: IL-10). Results are expressed as mean ± SD, NS, no significant; *P < 0.05; **P < 0.01; ***P < 0.001.

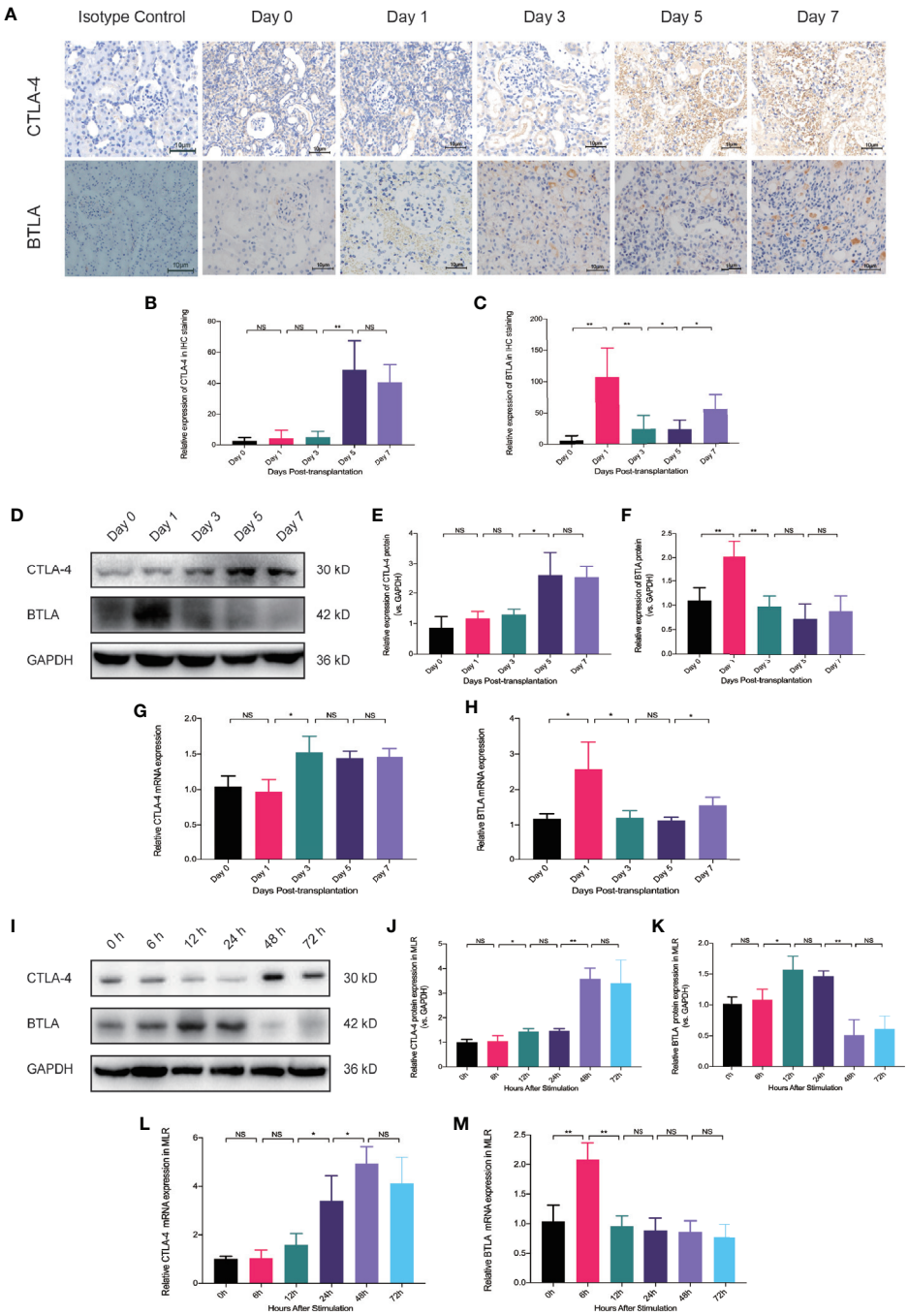

FIGURE 6 | The timing of CTLA-4 and BTLA expression upregulation in antigen-specific immune responses is different. **(A)** Immunohistochemical staining analysis of CTLA-4 and BTLA in renal tissues at different postoperative time points. Magnification: 400×. **(B)** Analysis of relative expression of CTLA-4 in the grafted kidney by IOD values. **(C)** Relative expression of BTLA in immunohistochemical staining. **(D)** Expression levels of CTLA-4 and BTLA in total graft protein from recipients at different postoperative time-points were analyzed by western blot. **(E)** Relative expression of CTLA-4 protein in kidney graft. **(F)** Relative expression of BTLA in total graft protein. **(G)** Analysis of relative expression of CTLA-4 mRNA in the postoperative recipient at different time points by qRT-PCT. **(H)** Relative expression levels of BTLA mRNA in transplanted kidney tissue. **(I)** Western blot analysis of CTLA-4 and BTLA protein expression in mixed lymphocytes reaction (MLR) after 0, 6, 12, 24, 48, and 72 h. **(J)** Analysis of relative expression of CTLA-4 protein in MLR receptor cells. **(K)** Analysis of relative BTLA protein expression in receptor lymphocytes. **(L)** qRT-PCR analysis was used to detect the relative expression of CTLA-4 mRNA in MLR at different times. **(M)** qRT-PCR analysis was used to detect the relative expression level of BTLA mRNA. Results are expressed as mean ± SD, NS, no significant; *P < 0.05; **P < 0.01.

improvement in renal function and prolongation of graft survival time compared to the monotherapy, suggesting a more favorable effect of coadministration against early postoperative acute rejection and possibility of inducing immune tolerance. We performed further pathological analysis showing that mixed acute rejection was attenuated with a reduction of C4d deposition in the combined treatment group. Furthermore, combination therapy decreased the infiltration of CD138+ cells in grafts, accompanied by downregulation of circulating DSA production. Similar to our previous work (26), BTLA overexpression reduced the generation of intimal arteritis in the graft and effectively suppressed TCMR. Interestingly, we found that Belatacept had an inhibitory effect on the ABMR but a weaker therapeutic effect on TCMR such as arterial endarteritis than single targeting BTLA. There has been less research on the function of Belatacept in ABMR (39), and recent studies showed that it appears to block the CD28-mediated activation of T follicular helper cells (Tfhs), thereby modulating B cells and reducing DSA production (40). A primate modeling study also reported a disruption of the germinal center by Belatacept (41). In the clinic, *de novo* DSA development in the phase III BENEFIT and BENEFIT-EXT studies showed that Belatacept-based immunosuppression is associated with a significantly lower incidence of *de novo* DSA development relative to cyclosporine-based immunosuppression over 7 years (84 months) of follow-up (42). These findings are similar to those we found, where Belatacept had a potential inhibitory effect on ABMR.

By observing T cell expression, we found that the nondifference in CD8+ cells between experimental groups and the significant change in CD4+ cells suggested that the combination therapy exerts inhibition mainly through regulating CD4+ T cells, thereby significantly reducing the frequency of circulating CD3+ cells and thus the infiltration of CD3+ T cells in grafts. The obvious effect of BTLA on T cells also explains its potent inhibition of TCMR. Serum cytokine levels were detected to understand the altered CD4+ cell differentiation in circulation. During acute rejection, naïve CD4+ T cells, stimulated by donor antigens, mainly differentiate into Th1 and Th2 cells. Th1 cells participate in rejection by secreting the inflammatory cytokines IL-2 and IFN-γ, which can also respond to the degree of T cell immune response (43). Th2 cells that secrete IL-4 and IL-10 are thought to have a dual role of anti-inflammatory and rejection suppression and are involved in inducing immune tolerance (44, 45). In our study, we found that Belatacept inhibited the secretion of IL-2 and IFN-γ more weakly than targeting BTLA but increased IL-4 and IL-10 production. This finding also suggests that Belatacept probably reduces Th1 cells and increases Th2 cells, while BTLA overexpression probably significantly inhibits Th1 cells, thereby reducing the proportion of CD4+ T cells. The shift of Th1 cells to Th2 cells is known as one of the mechanisms for constructing graft immune tolerance (46). Based on the changes in cytokine expression levels, we speculate that the combination treatment may downregulate Th1 cells and increase Th2 cell differentiation, causing Th1/Th2 shifts with prolonged graft survival. In addition, Foxp3 acts as a marker for regulatory T cells (Tregs) and has a protective effect on infiltration in immune organs. Foxp3+ T follicular regulatory cells (Tfrs) have recently been found to inhibit the onset of ABMR promoted by Tfh cells (47, 48). The upregulation of splenic Foxp3 in the Allo+Combination group also revealed a possible alteration in regulatory T cell differentiation. Therefore, Belatacept combined with BTLA overexpression may alter CD4+ T cell differentiation, affect the Th1/Th2 cell shift, promote regulatory T cell production, and thus inhibit acute rejection after renal transplantation.

After naïve CD4+ T cells are stimulated with antigen, Th1 differentiation predominates in the early stages, followed by Th2 cells that begin to secrete cytokines and exert inhibitory effects (49, 50). Similar to other studies, CTLA-4 expression began to increase after 24–48 h of antigenic stimulation *in vitro*, whereas BTLA expression was stimulated in cell culture at 6–12 h. In acute rejection after kidney transplantation, high expression of CTLA-4 was observed from postoperative day 5. The expression of BTLA is increased on postoperative day 1 and then rapidly decreases. These results suggested that BTLA acts as an early indicator of acute rejection and may exert its inhibitory effects earlier than CTLA-4. Based on these observations, we speculated that BTLA acts early in the acute rejection, possibly by effectively inhibiting Th1 cell differentiation, suppressing T cell proliferation and exhibiting a significant anti-TCMR effect. CTLA-4 was elevated later than BTLA, manifesting as inhibition of Th1 cells and stimulation of Th2 cell differentiation. This also revealed that different costimulatory molecules may have different action times in the antigen-stimulated immune response, with different mechanisms of action. Combination therapy has a better inhibitory effect on early acute rejection than single therapy.

Considering the potent effect of overexpressed BTLA on TCMR, it is possible that Belatacept provided a synergistic inhibitory effect, which offers hope for an immunosuppressive regimen free of CNIs. However, this still needs to be further verified in future comparative studies. Additionally, combination therapy effectively suppressed mixed rejection and can improve graft survival in patients with a risk of poor prognosis due to misdiagnosis, as TCMR or ABMR alone is an inadequate immunosuppressive therapy (51, 52). Combination therapy significantly prolonged the immunosuppressive state, suggesting the potential benefit of reducing the immunosuppressive dosage, minimizing drug toxicity and reducing the incidence of adverse events.

CONCLUSION

Overall, in both *in vivo* and *in vitro* experiments, we found that Belatacept reduced the production of DSA and had a probable inhibitory effect on acute ABMR after kidney transplantation. Belatacept combined with BTLA overexpression prolonged graft survival possibly by regulating circulating T cell differentiation, causing a Th1/Th2 cell shift, reducing T cell and plasma cell infiltration and inhibiting acute rejection. CTLA-4 and BTLA may explain the different effects of targeted therapy on T cell differentiation by their different durations of action in the immune response. In brief, Belatacept combined with BTLA

overexpression can attenuate acute rejection after kidney transplantation and prolong graft survival, which provides new ideas for the optimization of early immunosuppression protocols after clinical renal transplantation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Nanjing Medical University.

AUTHOR CONTRIBUTIONS

MG, HZ, and ZW conceived and designed this study. HZ, JZ, ZG, and HY performed the experiments and collected the data. HZ, ZW, JZ, HC, RT, and AC completed the data analysis and interpretation. AC provided essential suggestions for the data presentation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.618737/full#supplementary-material

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REVIEW
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The Role of Immune Checkpoint Molecules for Relapse After Allogeneic Hematopoietic Cell Transplantation

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Immune checkpoint molecules represent physiological brakes of the immune system that are essential for the maintenance of immune homeostasis and prevention of autoimmunity. By inhibiting these negative regulators of the immune response, immune checkpoint blockade can increase anti-tumor immunity, but has been primarily successful in solid cancer therapy and Hodgkin lymphoma so far. Allogeneic hematopoietic cell transplantation (allo-HCT) is a well-established cellular immunotherapy option with the potential to cure hematological cancers, but relapse remains a major obstacle. Relapse after allo-HCT is mainly thought to be attributable to loss of the graft-versus-leukemia (GVL) effect and hence escape of tumor cells from the allogeneic immune response. One potential mechanism of immune escape from the GVL effect is the inhibition of allogeneic T cells via engagement of inhibitory receptors on their surface including PD-1, CTLA-4, TIM3, and others. This review provides an overview of current evidence for a role of immune checkpoint molecules for relapse and its treatment after allo-HCT, as well as discussion of the immune mediated side effect graft-vs.-host disease. We discuss the expression of different immune checkpoint molecules on leukemia cells and T cells in patients undergoing allo-HCT. Furthermore, we review mechanistic insights gained from preclinical studies and summarize clinical trials assessing immune checkpoint blockade for relapse after allo-HCT.

Keywords: allogeneic hematopoietic cell transplantation, immune checkpoint, immune checkpoint inhibitor, anti-PD-1, graft-versus-host disease, graft-versus-leukemia

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INTRODUCTION

Our immune system is an important defense mechanism against invading pathogens as well as against cells that become malignant. Therefore, immunotherapy has become a significant pillar of cancer therapy. The first cellular immunotherapy was established in the 1950s, when Thomas et al. (1) performed the first successful allogeneic hematopoietic cell transplantation (allo-HCT). More recently, blocking physiological control mechanisms of the immune system with immune checkpoint inhibitors (ICI) has led to another major breakthrough in cancer immunotherapy (2). So far, ICI have shown the best clinical responses in patients with solid tumors, while clinical efficacy

in most hematological malignancies was lower. However, the possibility to enhance the graft-versus-leukemia (GVL) effect after allo-HCT with ICI has become an enticing concept in the past years. The combination of allo-HCT with ICI is an area of active investigation, which we will discuss in this review.

Allo-HCT, Graft-versus-Host Disease, and the Graft-versus-Leukemia Effect

Allo-HCT is a potentially curative therapy for diverse benign and high-risk malignant hematological diseases. The most frequent indications for allo-HCT are acute myeloid leukemia (AML), myeloid dysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and acute lymphocytic leukemia (ALL) (3, 4). An important element for the therapeutic success of allo-HCT is the recognition and elimination of residual malignant cells by allogeneic T cells present in the graft, commonly known as the GVL effect (5). However, the allogeneic donor T cells can also attack healthy tissues of the allo-HCT recipient, most frequently the skin, gastrointestinal tract, and liver. This results in one of the major and potentially lethal complications of allo-HCT, acute graft-versus-host disease (GVHD), which occurs in ca. 30–50% of allo-HCT recipients (6). Furthermore, tumor control by the allogeneic T cells is not extensive and durable enough in all patients. Loss of the GVL effect is thought to be one of the major reasons for relapse of primary disease, which remains the most common cause of death and treatment failure post allo-HCT (3, 7). Therefore, a current major objective is to reinstate the GVL effect without inducing or aggravating GVHD in patients relapsing post allo-HCT. One potential cellular therapy that is currently used to treat relapse after allo-HCT is the infusion of donor lymphocytes (DLI); however, its efficacy and toxicity vary across studies (8, 9). With the clinical breakthrough of immune checkpoint inhibitors (ICI), boosting the GVL effect with ICI post allo-HCT became a tempting concept.

ICI and Immune Related Adverse Events

Immune checkpoints are physiological control mechanisms of our immune system, which are crucial for maintaining immune homeostasis and the prevention of autoimmune reactions (10). As a general concept, inhibitory immunoreceptors expressed on the surface of T cells interact with specific ligands leading to reduced T cell activation and/or T cell apoptosis. The inhibitory checkpoint ligands can be expressed on stromal cells or antigen-presenting cells (APC) but also on tumor cells, which exploit these regulatory mechanisms to escape the anti-tumor immune response (11). In recent years, various different inhibitory immuno-receptors, also known as immune checkpoints, have been identified and analyzed for their role in cancer, including but not limited to PD-1, CTLA-4, LAG3, TIM3, TIGIT, and BTLA (summarized in **Figure 1**).

Inhibition of the two best described immune checkpoints, CTLA-4 and PD-1, using monoclonal antibodies has led to a breakthrough in cancer immunotherapy in the recent decade, showing remarkable responses and improved overall survival (OS) in many different solid tumors (24–27). Blocking the interaction of CTLA-4 and its ligands from the B7 family (CD80/CD86) using antibodies had shown first promising anti-tumor effects in murine cancer models in 1996 (28). Similarly, early studies demonstrated that interaction of PD-1 and its ligand PD-L1 on tumor cells represents a tumor immune escape mechanism and that blockade of the PD-1/PD-L1 axis reduced tumor growth in experimental models (29). These reports set the cornerstone for today's rapid clinical successes in the field of immune checkpoint blockade. To date, multiple immune checkpoint inhibitors (blocking either CTLA-4, PD-1, or PD-L1) are approved for more than 15 different cancer entities, however, efficacy has so far been most promising in solid tumors (**Table 1**). By systemically increasing T cell activity, ICI can also enhance autoimmune responses and induce inflammatory side effects, which are termed immune-related adverse events (irAEs) (30). These are more common and severe with CTLA-4 blockade than with PD-1/PD-L1 blockade and can principally affect any organ system (30–32). IrAEs can be life-threatening, but are usually well manageable with steroid treatment (31). Nevertheless, both irAEs and GVHD are complications that require close monitoring when combining ICI with allo-HCT and will be topics covered in this review.

IMMUNE CHECKPOINTS AND RELAPSE AFTER ALLO-HCT

Expression of Immune Checkpoint Ligands on Leukemia Cells in Patients Undergoing allo-HCT

Relapse after allo-HCT is thought to be attributed mainly to the loss of the GVL effect and hence the escape of tumor cells from the allogeneic immune response. Various different mechanisms of immune escape from the GVL effect post allo-HCT exist, which have recently been reviewed (33). These include downregulation of MHC molecules, production of anti-inflammatory factors and metabolically active enzymes, loss of pro-inflammatory cytokine production, and notably the expression of immune checkpoint ligands (33). Upregulation of immune regulatory molecules on AML blasts has been shown to be a distinctive characteristic and driver of AML relapse post allo-HCT (34). Already in 2011, a study focusing on the PD-1/PD-L1 axis reported increased PD-L1 expression on myeloid leukemia cells after IFNγ and TNFα stimulation as well as PD-1 expression on minor histocompatibility antigen (MiHA)-specific memory CD8 T cells (35). Subsequently, comprehensive immuno-phenotyping of AML blasts before and after allo-HCT revealed an upregulation of PD-L1, B7-H3, poliovirus receptor-related 2 (PVRL2/CD112, ligand for TIGIT) and CD80 at relapse after allo-HCT compared to initial diagnosis (34). Concomitantly, the percentage of PD-1 expressing T cells was higher at post-transplantation relapse than in healthy controls and in AML patients before allo-HCT. To investigate the functional relevance of these findings, the authors used co-culture experiments of leukemia blasts and donor-derived T cells from one patient. *Ex vivo* addition of anti-PD-L1 blocking antibody caused increased T cell proliferation and IFNγ production, indicating that in some patients with deregulated

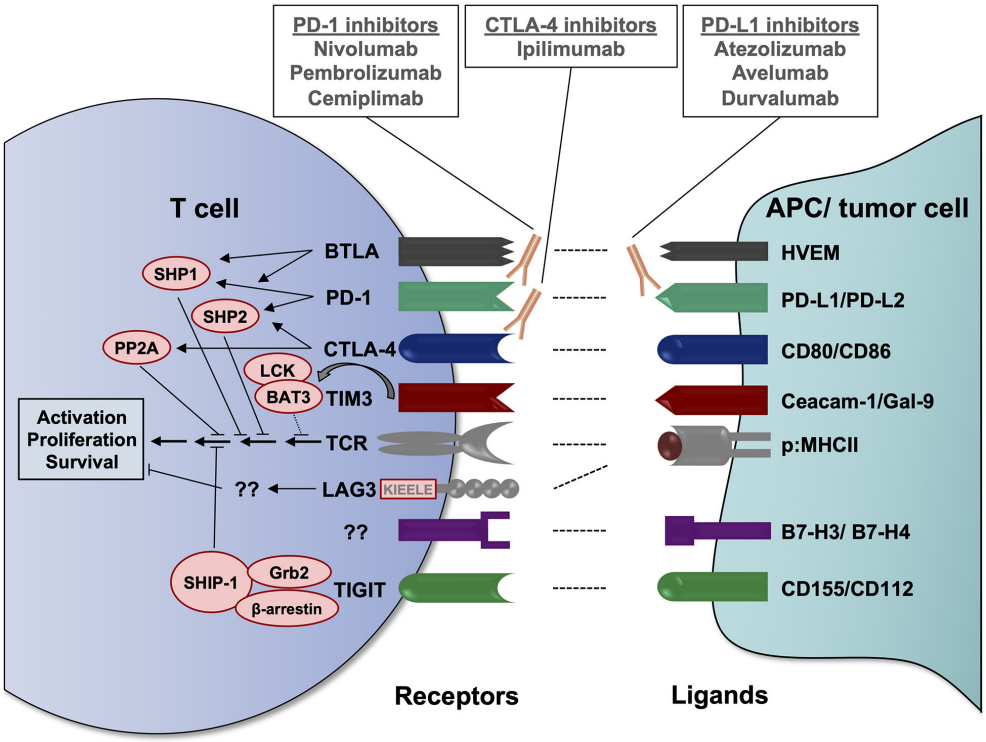

FIGURE 1 | Important inhibitory immunoreceptors (immune checkpoints), their ligands and known signaling mechanisms. Immune checkpoints expressed on the T cell surface bind to their respective ligands on antigen-presenting cells (APC) or tumor cells, resulting in an inhibitory signal to the T cell. These interactions may occur in lymph nodes at the initiation of T cell responses or in peripheral tissues/tumor sites during the T cell effector response. Via its immunoreceptor tyrosine-based inhibitory motif (ITIM) and its immunoreceptor tyrosine-based switch motif (ITSM), PD-1 can recruit the SH2 domain-containing tyrosine phosphatases SHP1 and SHP2, which dephosphorylate TCR signaling mediators, including ZAP70 and Lck, as well as CD28 signaling mediators (12, 13). CTLA-4 competes with CD28 for the binding of B7 molecules, preventing the CD28 co-stimulatory signal, and recruits phosphatases that counteract T cell activation, including SHP2 and protein phosphatase 2A (PP2A) (12, 14). Similar to PD-1, BTLA can recruit the phosphatases SHP1 and SHP2 to inhibit TCR and CD28 downstream signaling (15, 16). Upon T cell activation and in the absence of TIM3 ligands, HLA-B-associated transcript 3 (BAT3) is bound to the cytoplasmic domain of TIM3 and to the tyrosine kinase LCK, which is involved in proximal TCR signaling. Upon binding of its ligands Ceacam-1 or gal-9, tyrosine residues in the intracellular domain of TIM3 are phosphorylated, causing the release of BAT3 from its cytoplasmic tail and recruitment of tyrosine phosphatases, contributing to TCR signaling inhibition (17–19). LAG3 does not contain an ITIM or ITSM inhibitory motif, but different well-conserved amino acid motifs, including a KIEELE motif, which potentially contribute to its T cell inhibitory function (20, 21). The exaxt signaling mechanisms remain to be elucidated. Upon binding of its ligands CD155 (PVR) or CD112 (PVRL2), TIGIT has been proposed to recruit the adapter proteins Grb2 and β-arrestin and the SH2-containing inositol phosphatase-1 (SHIP-1), which interferes with phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and NF-κB signaling (22, 23). However, these findings have been made in NK cells and have yet to be confirmed in the T cell setting. BAT3, HLA-B-associated transcript 3; BTLA, B and T lymphocyte attenuator; Ceacam-1, carcinoembryonic antigen-related cell adhesion molecule-1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; Gal-9, galectin 9; Grb2, growth factor receptor-bound protein 2; HVEM, herpesvirus entry mediator; LAG3, lymphocyte activation gene-3; p:MHCII, peptide:major histocompatibility complex II; PD-1, programmed cell death protein 1; PD-L, PD-1 ligand; PP2A, protein phosphatase 2A; PVRL2, poliovirus receptor-related 2; SHIP-1, SH2-containing inositol phosphatase-1; SHP, SH2 domain-containing tyrosine phosphatase; TCR, T cell receptor; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIM3, T cell immunoglobulin and mucin-domain containing protein 3.

PD-1/PD-L1 expression, checkpoint inhibition might reinstate the GVL effect against relapsed AML (34).

Expression of Inhibitory Checkpoint Receptors on T Cells in Patients Undergoing allo-HCT

An increasing number of studies report on the co-expression of inhibitory checkpoint receptors on donor T cells and their correlation with relapse post allo-HCT. Jain and colleagues found that PD-1 expression was elevated both on peripheral blood (PB) T cells from relapsed as well as non-relapsed patients having undergone human leukocyte antigen (HLA)-matched stem cell transplantation. This indicates that PD-1 is not the sole predominant marker for leukemia-specific T cell exhaustion in patients relapsing after allo-HCT (36). Deeper analyses using single-cell RNA sequencing of one patient sample revealed that LAG3 and TIM3 were overexpressed in leukemia antigen-specific T cells (36). In line with these data, the frequency of peripheral blood PD-1-high TIM3$^+$ T cells was strongly associated with leukemia relapse in 11 AML patients who received allo-HCT (37). Importantly, the PD-1-high TIM3$^+$ cells showed functional signs of exhaustion, including reduced production of IL-2, IFNγ and TNFα, and their increase occurred before clinical

TABLE 1 | Currently approved immune checkpoint inhibitors (ICI) for cancer immunotherapy.

Name of ICI	Target	FDA-approved indications Solid tumors	FDA-approved indications Hematological malignancies
Ipilimumab (Yervoy®)	CTLA-4	• Melanoma	
Nivolumab (Opdivo®)	PD-1	• Melanoma • NSCLC • SCLC • Renal cell carcinoma • Squamous cell carcinoma of the head and neck • Urothelial carcinoma • Hepatocellular carcinoma • Esophageal squamous cell carcinoma	• Classical HL
Pembrolizumab (Keytruda®)	PD-1	• Melanoma • NSCLC • SCLC • Renal cell carcinoma • Head and neck squamous cell cancer • Urothelial carcinoma • Gastric cancer • Esophageal cancer • Cervical cancer • Endometrial carcinoma • Hepatocellular carcinoma • Merkel cell carcinoma • Microsatellite Instability-High (MSI-H) or mismatch repair deficient cancer* • Tumor Mutational Burden-High* (TMB-H) cancer • Cutaneous squamous cell carcinoma	• Classical HL • Primary mediastinal large B cell lymphoma
Cemiplimab (Libtayo®)	PD-1	• Cutaneous squamous cell carcinoma	
Ipilimumab + Nivolumab	CTLA-4 + PD-1	• Melanoma • Renal cell carcinoma • Metastatic colorectal cancer • Hepatocellular carcinoma • NSCLC • Malignant pleural mesothelioma	
Atezolizumab (Tecentriq®)	PD-L1	• Melanoma • Urothelial carcinoma • NSCLC • SCLC • Triple-negative breast cancer • Hepatocellular carcinoma	
Avelumab (Bavencio®)	PD-L1	• Urothelial carcinoma • Renal cell carcinoma • Merkel cell carcinoma	
Durvalumab (Imfinzi®)	PD-L1	• Urothelial carcinoma • NSCLC • SCLC	

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HL, Hodgkin lymphoma; ICI, immune checkpoint inhibitor; NSCLC, non-small lung cancer; PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand 1; SCLC, small cell lung cancer.
**Limitation: The safety and effectiveness of pembrolizumab in pediatric patients with MSI-H/TMB-H central nervous system cancers have not been established.*

diagnosis of leukemia relapse, suggesting their predictive value (37). Similarly, Williams et al. (38) reported a trend toward a higher frequency of $CD8^+PD\text{-}1^+TIM3^+$ T cells and $CD8^+PD\text{-}1^+LAG3^+$ T cells in the bone marrow (BM) of AML patients with relapse. These findings were confirmed in a study involving 32 AML patients relapsing or maintaining complete remission (CR) after allo-HCT (39). In the BM of relapsing patients, a higher proportion of $CD8^+$ T cells expressed CTLA-4, PD-1 and TIM3 when compared to CR patients (39). This was only the case in patients who underwent HLA-identical allo-HCT, while the profile of inhibitory receptors did not correlate with clinical outcome after haploidentical transplantation, hypothetically due to the higher degree of HLA-mismatch and therefore and increased inflammatory cytokine milieu. Of note, the inhibitory receptor expressing T cells displayed a skewed T cell receptor (TCR) repertoire at relapse and better recognized and eliminated matched leukemic blasts *in vitro* when compared to inhibitory receptor negative T cells, indicating that inhibitory receptor expression marks leukemia-specific T cells (39). In agreement with this hypothesis, PD-1, TIGIT, and KLRG-1 were highly co-expressed on circulating MiHA-reactive CD8 T cells after allo-HCT and this expression was associated with relapse risk (40). A further study by Hattori et al. (41) confirmed that a high expression of TIGIT in BM samples of AML patients after allo-HCT correlated with poor overall survival (OS) and progression-free survival (PFS) as well as decreased incidence of acute GVHD, indicating a regulatory effect of TIGIT on allo-reactive cells.

In addition to its expression on exhausted T cells, TIM3 is a marker for acute myeloid leukemia stem cells (LSCs), which discriminates these cells from normal hematopoietic stem cells (42, 43). In a cohort of 57 AML patients treated with allo-HCT, high percentages of $TIM3^+$ LSC at engraftment were a significant independent risk factor for relapse after allo-HCT (44).

Preclinical ICI Animal Studies

CTLA-4

The T cell surface molecules CD28 and CTLA-4 are structurally related and both molecules bind to B7-1 (CD80) and B7-2 (CD86), transmitting T cell stimulatory and inhibitory downstream signals, respectively (45). An early study by Blazar et al. (46) showed that blockade of CTLA-4 at an early

time point during allo-HCT augmented alloreactivity, resulting in accelerated GVHD lethality in a major histocompatibility complex (MHC) mismatched mouse model of bone marrow transplantation (BMT). In contrast, treatment with anti-CTLA-4 mAb at a later time point post-BMT in the context of DLI strongly enhanced the GVL effect, while only mildly increasing GVHD (46). Delayed CTLA-4 blockade induced a host-derived anti-leukemic effect in a MiHA-mismatched BMT mouse model, while not inducing GVHD, but an autoimmune syndrome with autoimmune hepatitis and circulating anti-DNA auto-antibodies (47). Importantly, both the anti-leukemic effect and the autoimmune pathology were mediated by host and not donor T cells, but depended on the allogeneic component, as neither effect was seen after syngeneic BMT (47).

PD-1/PD-L1/2 Axis

Numerous studies have addressed the question of how PD-1 and its ligands PD-L1 and PD-L2 regulate the delicate balance between GVHD and GVL post-allo-HCT. Already in 2003, Blazar et al. (48) demonstrated in murine models that blocking either PD-1 or PD-L1 aggravates GVHD in an IFNγ dependent mechanism (48). Blocking both CTLA-4 and PD-1 together was additive in enhancing GVHD, indicating the non-redundancy of these pathways. In a follow-up study, they identified the PD-1/PD-L1 axis to be predominant in regulating GVHD development, as compared to PD-1/PD-L2 interaction, and that PD-L1 expression on host parenchymal cells is critical for the suppression of acute GVHD (49). However, the effects of PD-1/PD-L1/PD-L2 blockade on the GVL response were not assessed in these studies.

Asakura and colleagues demonstrated that blocking PD-L1 antibody treatment early after allo-HCT improved T cell effector functions and GVL activity in mice, but this occurred at the expense of aggravated GVHD (50). In contrast, *in vivo* PD-L1 blockade at later time points after DLI (day 48–60) was able to enhance cytotoxic T lymphocyte (CTL) activity and GVL effects without induction of GVHD (51). Similarly, the efficacy of adoptive transfer of gene-modified leukemia-specific T cells late (56 days) after T cell-depleted BM transplantation, could be enhanced by additional systemic blockade of PD-L1, without inducing GVHD (52). Michonneau et al. (53) identified the differentiation of GVHD and GVL responses by anatomical differences in CTL activity and PD-L1/PD-L2 expression in a mouse model of single MiHA-mismatched allo-HCT. PD-1 ligand expression was low on liver antigen-presenting cells (APCs) and high on APCs and endothelial cells in the lymph nodes, resulting in GVHD development and local tumor immune escape, respectively. PD-1 blockade was able to restore CTL killing activity in lymph nodes, together indicating that the PD-1 pathway is not equally engaged in all organs (53). Further work by the Blazar group revealed that, in contrast to host PD-L1 expression, PD-L1 expression on donor T cells augments GVHD in murine allo-HCT models (54). *Pdl1* deficient donor T cells caused reduced GVHD, while they importantly still displayed potent GVL function, suggesting that selective inhibition of PD-L1 on donor T cells might ameliorate GVHD, while preserving the GVL effect (54). Taken together, these studies indicate a time-, organ- and cell type-dependent function of the PD-1/PD-L1/PD-L2 axis during allo-HCT.

A recent study assessed the mechanisms of GVL failure using an elegant mouse model, in which GVL is exclusively mediated by alloreactive $CD8^+$ T cells recognizing the MiHA H60, making it possible to specifically track and analyze the GVL-inducing T cells (55, 56). Next to insufficient H60 presentation, the GVL effect in this model failed due to the development of leukemia-specific T cell exhaustion, characterized by expression of the inhibitory receptors PD-1, TIGIT, LAG3, and TIM3 and the transcription factor TOX, which has recently been shown to drive T cell exhaustion (57–61). Blockade of PD-1 was able to reverse the T cell exhaustion phenotype and restore the GVL effect, whereas blockade of TIM3, LAG3, and TIGIT were not, suggesting that PD-1 may be the dominant inhibitory checkpoint contributing to GVL failure in mice (55).

Clinical Evidence

Translating the above-described preclinical evidence into clinical application of ICI for patients relapsing after allo-HCT has been challenging, due to understandable concern regarding the occurrence of immune-related side effects, in particular severe GVHD. To date there is only limited data regarding the efficacy of checkpoint inhibitors before or after allo-HCT in hematological malignancies other than Hodgkin lymphoma (HL). In the following paragraphs, we focus on clinical trials that have assessed CTLA-4 or PD-1 blockade in patients relapsing after allo-HCT. Major studies evaluating ICI therapy in hematological malignancies relapsing after allo-HCT are summarized in **Table 2**.

CTLA-4 Blockade Post allo-HCT

An early dose escalation trial by Bashey et al. (70) demonstrated an acceptable safety profile of ipilimumab in 29 patients with malignancies that were recurrent or progressive after allo-HCT. The underlying disease of the majority of patients was HL (48%) or multiple myeloma (21%). A single infusion of ipilimumab at doses from 0.1 up to 3 mg/kg did not result in acute or chronic GVHD induction, while four patients developed irAEs. However, it has to be noted that patients with prior grade 3 or 4 acute GVHD development were excluded from this study. Three patients with lymphoid malignancies demonstrated objective disease responses after a single dose of 1 or 3 mg/kg ipilimumab (70). In a proportion of the patients, increases in activated $CD4^+$ T cells were observed after ipilimumab infusion (71).

A subsequent phase I/Ib study analyzed safety and efficacy of ipilimumab in 28 patients with hematological malignancies relapsing after allo-HCT with no history of prior grade 3 or 4 acute GVHD (64). Ipilimumab dosage was 3 or 10 mg/kg every 3 weeks for a total of 4 doses, with additional doses every 12 weeks for up to 60 weeks in patients with clinical benefit. Response to treatment was dose-dependent, with no response observed in patients who received a dose of 3 mg/kg, while in the 10 mg/kg cohort ($n = 22$) 23% of patients achieved a CR and 9% a PR. GVHD that led to treatment discontinuation, but was responsive to glucocorticoids, occurred in 4 patients, and irAEs, including

TABLE 2 | Selected clinical trials of checkpoint inhibitor therapy in hematological malignancies following allo-HCT.

References	Intervention	Study population	Study type	Outcome
Herbaux et al. (62)	Nivolumab (q2w, 3 mg/kg)	HL relapsed after allo-HCT ($n = 20$)	Retrospective study	ORR/CR/PR = 95/42/52% 12 month PFS/OS = 58.2/78.7%
Haverkos et al. (63)	Nivolumab (q2w, 3 mg/kg): $n = 28$ Pembrolizumab (q3w, 200 mg): $n = 3$	Lymphoma relapsed after allo-HCT ($n = 31$) HL: $n = 29$; FL + HL: $n = 1$; transformed FL: $n = 1$	Retrospective study	ORR/CR/PR = 77/50/27% Median PFS/OS = 19 months/not reached
Davids et al. (64, 65)	Ipilimumab (q3w) 3 mg/kg: $n = 6$ 5 mg/kg: $n = 15$ 10 mg/kg: $n = 22$	Hematological malignancies relapsed after allo-HCT ($n = 43$) AML: $n = 18$; HL: $n = 7$; NHL: $n = 5$; CLL: $n = 3$; MM: $n = 3$; MDS: $n = 3$; ALL: $n = 2$; MPN: $n = 1$; CMML: $n = 1$	Phase I/Ib	3 mg/kg: no response 5 mg/kg: ORR/CR/PR = 23/0/23% median PFS/OS = 3.4/7 months 10 mg/kg: ORR/CR/PR = 32/23/9% median PFS/OS = 9.4/28.3 months
Khouri et al. (66)	Lenalidomide (10 mg/day for 21 days) + Ipilimumab (3 mg/kg, single dose) Repeated for 2 cycles	Lymphoid malignancies relapsed after allo-HCT ($n = 19$) MCL: $n = 3$; CLL: $n = 2$; FL: $n = 2$; THL: $n = 1$; DLBCL: $n = 1$; ALCL: $n = 1$	Phase II	ORR/CR/PR = 70/40/30% 90% OS at median follow-up of 20.5 months
Holderried et al. (67)	Ipilimumab ($n = 10$) Nivolumab ($n = 5$) Nivolumab + DLI ($n = 5$) Nivolumab + Ipilimumab ($n = 1$)	Hematological malignancies relapsed after allo-HCT ($n = 21$) MDS/AML: $n = 12$; NHL: $n = 5$; ALL: $n = 2$; MF: $n = 2$	Retrospective study	Overall ORR/CR/PR = 43/14/29% Ipilimumab: ORR = 20% Nivolumab: ORR = 40% Nivolumab + DLI: ORR = 80% Overall median OS = 79 days Ipilimumab: median OS = 39 days Nivolumab (±DLI): median OS = 282 days
Kline et al. (68)	Pembrolizumab (q3w, 200 mg)	Hematological malignancies relapsed after allo-HCT Interim analysis ($n = 11$) AML: $n = 8$; DLBCL: $n = 2$; HL: $n = 1$ Planned $n = 26$	Phase I	ORR/CR/PR = 29/29/0% (CR reached in 1 DLBCL and 1 HL patient)
Davids et al. (69)	Nivolumab (q2w) 1 mg/kg: $n = 6$ 0.5 mg/kg: $n = 22$	Hematological malignancies relapsed after allo-HCT ($n = 28$) AML: $n = 10$; MDS: $n = 7$; HL: $n = 5$; NHL: $n = 3$; CLL: $n = 1$; CMML: $n = 1$; Leukemia NOS: $n = 1$	Phase I/Ib	1 mg/kg: ORR/CR/PR = 50/17/33% 0.5 mg/kg: ORR/CR/PR = 23/0/23% median PFS/OS = 3.7/21.4 months

ALCL, anaplastic large T-cell lymphoma; allo-HCT, allogeneic hematopoietic cell transplantation; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CR, complete remission; DLBCL, diffuse large B-cell lymphoma; DLI, donor lymphocyte infusion; FL, follicular lymphoma; HL, Hodgkin lymphoma; MCL, mantle cell lymphoma; MDS, myelodysplastic syndrome; MF, myelofibosis; MM, multiple myeloma; MPN, myeloproliferative neoplasm; NHL, non-Hodgkin lymphoma; NOS, not otherwise specified; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PR, partial remission; THL, triple-hit lymphoma.

one fatality, were observed in 6 patients. At a median follow-up of 27 months, OS and PFS were 54 and 32% for the 10 mg/kg group, respectively (64). An update of this study reported about an intermediate dose (5 mg/kg) phase Ib extension cohort including 15 additional patients (65). At 5 mg/kg ipilimumab, partial responses were also observed, but the reduced dose did not improve the rate of GVHD or irAEs (65).

Furthermore, combination treatment of lenalidomide (10 mg/day for 21 days) followed by ipilimumab (3 mg/kg) in ten patients relapsing after allo-HCT has been assessed in a phase II trial (66). One patient with known GVHD history had a flare of his symptoms after the first lenalidomide cycle that precluded further treatment, while all others completed treatment without GVHD development. Overall response rate (ORR) was 70% (4 CR, 3 PR) and at a median follow-up time of 20.5 months 90% of patients were alive. Importantly, ipilimumab plus lenalidomide combination treatment led to significantly increased numbers of circulating $CD4^+ICOS^+FoxP3^-$ conventional T cells (66).

PD-1 Blockade Post allo-HCT

Hodgkin Lymphoma

Given the clinical success of anti-PD-1 therapy in HL, multiple early case reports and case series describing the use of anti-PD-1 antibodies in patients with HL relapsing after allo-HCT have been published. In these reports, some patients benefitted from anti-PD-1 therapy post allo-HCT without the occurrence of serious side effects [nivolumab (72–74) and pembrolizumab (75)], while other patients developed severe toxicity with fatalities from GVHD [nivolumab (76) and pembrolizumab (77, 78)].

Herbaux et al. (62) retrospectively assessed the efficacy and toxicity of nivolumab in 20 HL patients relapsing after allo-HCT. Response rates were high (ORR 95%, CR 42%, PR 52%) and 1-year PFS and OS were 58.2 and 78.7%, respectively. Acute

GVHD occurred in six patients (30%) within 1 week after the first nivolumab dose and was manageable with standard GVHD treatment. All six patients had prior history of acute GVHD. Time between allo-HCT and nivolumab treatment start was significantly shorter in patients developing GVHD (62). Another retrospective study by Haverkos et al. (63) revealed promising response rates but also high GVHD frequency after anti–PD-1 treatment. Thirty one patients with lymphoma relapse after allo-HCT were treated with nivolumab ($n = 28$) or pembrolizumab ($n = 3$), resulting in an ORR of 77% (15 CR, 8 PR). However, 55% of patients developed GVHD already after 1–2 doses of anti-PD-1 treatment, including grade III-IV GVHD in 9 patients and 8 deaths related to treatment-emergent GVHD (4 acute GVHD and 4 chronic GVHD) (63).

Hematological Malignancies Other Than HL

While the above-described studies mainly included patients with HL, there is increasing interest in the possibility to use checkpoint blockade in the context of myeloid malignancies relapsing post allo-HCT. In a retrospective multi-center study, 21 patients with malignancies other than HL ($n = 12$ MDS/AML, $n = 5$ NHL, $n = 2$ ALL, $n = 2$ myelofibrosis) relapsing after allo-HCT were treated with ICI (67). Patients received either nivolumab or ipilimumab alone, a combination of both, or a combination of nivolumab with DLI. The ORR was 43% (3 CR, 6 PR), with higher response rates observed in patients receiving nivolumab plus DLI (ORR = 80%) compared to patients receiving nivolumab alone (ORR = 40%) or ipilimumab alone (ORR = 20%). However, grade III/IV aGvHD or moderate/severe cGvHD developed in 29% of patients, of which 83% were steroid-refractory (67). Kline et al. (68) presented early results from a still recruiting phase I study of pembrolizumab for the treatment of AML, MDS or B cell lymphoma relapse following allo-HCT. However, in 8 patients with AML treated so far, pembrolizumab seemed to have only limited effect with a best response of SD observed in 2 patients (68).

Recently, data from the first prospective trial of nivolumab for relapsed hematological malignancies (myeloid $n = 19$, lymphoid $n = 9$) after allo-HCT were reported (69). Nivolumab was administered every 2 weeks starting with a 1 mg/kg cohort ($n = 6$), of which two experienced dose-limiting toxicity from irAEs, resulting in dose reduction to 0.5 mg/kg for the remaining 22 patients. Anti-tumor activity was only modest, with an ORR of 29% and 1-year PFS and OS of 23 and 56%, respectively. ORR was higher in patients with lymphoid malignancies (44%) as compared to patients with myeloid malignancies (21%). Chronic or acute GVHD occurred in 39% of patients and was fatal in two patients (69).

In a recent study, low-dose regimens of pembrolizumab and nivolumab in the post allo-HCT settings were tested in a small patient cohort. Two heavily pretreated patients with HL relapsing after allo-HCT received 40 mg of nivolumab every 2 weeks (79). One of them remained in CR at 22 months; the other remained in PR at 6 months at the time point of analysis. Both patients did not develop any irAEs (79). In contrast, another recent phase I study of low-dose nivolumab as maintenance therapy post allo-HCT reported on unexpected severe toxicities (80). Four patients with AML or MDS were treated with nivolumab at 1 mg/kg every 2 weeks for four doses. All of them developed irAEs, and two patients experienced serious adverse events, including grade 4 neutropenia and grade 3 autoimmune encephalopathy, resulting in study termination (80).

Taken as a whole, these studies indicate that lower doses of anti-PD-1 treatment might have the potential to induce responses without inducing severe immunological complications, but also highlight the need for further dose-finding studies, potentially resulting in differing optimal dosing regimens for different underlying malignancies. Overall, the studies so far suggest that frequency and severity of immune-related adverse events and GVHD are higher in anti-PD-1 treated patients than in anti-CTLA-4 treated patients in the post allo-HCT setting.

Ongoing Clinical Trials

Multiple phase I and phase II clinical trials of checkpoint inhibitor therapy following allo-HCT are currently ongoing (summarized in **Table 3**). Many of them focus not only on HL but on AML and MDS and both ICI monotherapy and combination therapies are studied. The results of these trials could give more insight into efficacy and safety of ICI in the post-transplantation settings in diseases other than HL and the results are eagerly anticipated.

DISCUSSION AND FUTURE PERSPECTIVES

Allo-HCT is a well-established cellular immunotherapy option with the potential to cure high-risk hematological malignancies. However, relapse remains the major cause of death and treatment failure after allo-HCT. By inhibiting negative regulators of the immune response, checkpoint blockade can increase anti-tumor immunity, but has been primarily successful in solid cancer therapy so far.

On the one hand, boosting the allogeneic immune response post allo-HCT by blocking immune checkpoints is an appealing concept to prevent or treat relapse of hematological cancers. Numerous studies have found a connection between the expression of inhibitory checkpoints and disease relapse post allo-HCT. Clinical trials indicate therapeutic potential for the combination of these two immunotherapies, although lymphoid malignancies seem to be more responsive than myeloid malignancies thus far. Future preclinical studies and clinical trials will be crucial to further assess which checkpoints are the best therapeutic targets, taking into consideration the underlying disease, risk of side effects, optimal dose, timing, and therapy duration. The results of ongoing studies focusing on myeloid malignancies and assessing dual checkpoint blockade post allo-HCT are eagerly awaited to answer these open questions. Furthermore, the increased expression of other immune checkpoints on T cells in murine GVL models and in patients relapsing after allo-HCT, including TIM3 and TIGIT, suggests that novel immune checkpoint inhibitors blocking these molecules might offer potential treatment options post allo-HCT.

TABLE 3 | Selected ongoing clinical trials of checkpoint inhibitor therapy in hematological malignancies following allo-HCT.

Clinical trial identifier	Intervention	Study population	Phase	Planned n	Study start	Status
ICI monotherapy						
NCT03146468	Nivolumab	Relapsed/residual hematological malignancies after allo-HCT	II	14	May 2017	Active, not recruiting
NCT02981914 (68)	Pembrolizumab	AML/MDS/B cell lymphoma relapsed after allo-HCT	I	26	Mar 2017	Recruiting
NCT03286114	Pembrolizumab	AML/ALL/MDS relapsed after allo-HCT	I/Ib	20	December 2017	Recruiting
2017-002194-18 (EudraCT)	Nivolumab	Relapse of AML after allo-HCT	I/II	20	March 2018	Active, not recruiting
NCT04361058	Nivolumab	High risk AML/MDS relapsed after arm A: HLA-matched unrelated donor allo-HCT arm B: HLA-haploidentical allo-HCT	I	36	April 2020	Recruiting
ICI combination therapy						
NCT02846376	Nivolumab vs. Ipilimumab vs. Nivolumab + Ipilimumab	AML/MDS at risk for relapse after allo-HCT	I	8	March 2019	Active, not recruiting
NCT03600155	Nivolumab vs. Ipilimumab vs. Nivolumab + Ipilimumab	AML/MDS relapsed/refractory after allo-HCT	Ib	55	October 2018	Recruiting
NCT04128020	Nivolumab + Azacitidine	AML/high risk MDS after reduced-intensity allo-HCT	I	48	October 2019	Recruiting

allo-HCT, allogeneic hematopoietic cell transplantation; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; HLA, human leukocyte antigen; ICI, immune checkpoint inhibitor; MDS, myelodysplastic syndrome.

On the other hand, both allo-HCT and ICI therapy commonly induce inflammatory side effects, referred to as GVHD and irAEs, respectively. Although the roots and pathogenesis of these complications are distinct (allo- vs. auto-immunity), some patho-mechanisms seem to be shared between them, potentially adding up if these therapies are combined. For example, we and others found that the microRNA miR-146a is involved in the regulation of both acute GVHD after allo-HCT and irAEs of ICI therapy (81–84), indicating shared regulatory pathways in these complications. Therefore, the monitoring of immunological complications is of high importance for patients treated with ICIs before or after allo-HCT. Potential strategies to prevent or manage GVHD and irAEs in the context of ICI include starting ICI treatment at a low dose (possibly followed by dose escalation), immediate discontinuation of ICI therapy in the event of severe toxicity and rapid treatment with corticosteroids. History of prior GVHD seems to be an adverse risk factor for subsequent GVHD and both preclinical and clinical data indicate that a shorter interval between allo-HCT and ICI therapy is associated with a higher risk of immunological complications (46, 50–52, 62), which should be taken into consideration before initiation of ICI treatment.

Clinical trials in the solid cancer setting suggested that severe development of severe irAEs was more frequent with ipilimumab compared to nivolumab (30, 31, 85–87). In contrast, frequency and severity of irAEs and GVHD seem to be slightly higher in anti-PD-1 treated patients than in anti-CTLA-4 treated patients in the post allo-HCT setting, although direct evidence from head-to-head comparisons of these two scenarios is lacking. Therefore, on the one hand, differences in study design and patient characteristics, including timing of ICI treatment post allo-HCT, graft source, GVHD prophylaxis, and history of prior GVHD, might be a reason for this discrepancy. On the other hand, the conditioning regimen, GVHD prophylaxis, allogeneic HSC transfer, and increased pro-inflammatory milieu post allo-HCT are important factors that influence the immune system and might account for differences in the ICI toxicity profile. Another issue might be the mechanistic differences between CTLA-4 and PD-1 blockade (12, 88). Since CTLA-4 plays a more important role in early immune responses within lymph nodes and the T cell priming process and PD-1 rather during later phases of the immune response, peripheral T cell activity and maintenance of self-tolerance, toxicity levels may be skewed in favor of CTLA-4-blockade in a context without alloreactivity, that is, solid tumors.

Future studies are required to further delineate the pathophysiological mechanisms and assess the prophylactic and treatment strategies to minimize irAE and GVHD development while preserving the therapeutic efficacy of ICI.

AUTHOR CONTRIBUTIONS

NK collected and reviewed literature, discussed the studies, and wrote the original draft of the manuscript. DAR, RK, and RZ contributed to writing and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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HLA Class I Molecules as Immune Checkpoints for NK Cell Alloreactivity and Anti-Viral Immunity in Kidney Transplantation

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Natural killer (NK) cells are innate lymphocytes that can kill diseased- or virally-infected cells, mediate antibody dependent cytotoxicity and produce type I immune-associated cytokines upon activation. NK cells also contribute to the allo-immune response upon kidney transplantation either by promoting allograft rejection through lysis of cells of the transplanted organ or by promoting alloreactive T cells. In addition, they protect against viral infections upon transplantation which may be especially relevant in patients receiving high dose immune suppression. NK cell activation is tightly regulated through the integrated balance of signaling *via* inhibitory- and activating receptors. HLA class I molecules are critical regulators of NK cell activation through the interaction with inhibitory- as well as activating NK cell receptors, hence, HLA molecules act as critical immune checkpoints for NK cells. In the current review, we evaluate how NK cell alloreactivity and anti-viral immunity are regulated by NK cell receptors belonging to the KIR family and interacting with classical HLA class I molecules, or by NKG2A/C and LILRB1/KIR2DL4 engaging non-classical HLA-E or -G. In addition, we provide an overview of the methods to determine genetic variation in these receptors and their HLA ligands.

Keywords: NK cell, solid organ transplantation, KIR, NKG2A, HLA class I

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INTRODUCTION

Kidney transplantation is considered to be the best treatment option for patients with end-stage renal failure since transplantation gives better survival outcome and improved quality of life compared to dialysis (1). After transplantation, a primary concern is the function of the allograft, which can fail at early or late stage post transplantation due to various complications including allograft rejection and the occurrence of infections.

Allograft rejection is the result of concerted actions of several immune effector cells. During allograft rejection, the immune cells of the recipient get activated by alloantigens of the donor leading to immune responses against the graft and subsequently pathological changes, that can destroy the graft if not controlled (2). Rejection can occur minutes or days after transplantation

(hyper acute rejection), weeks or months after transplantation (acute rejection) and months or years after transplantation (chronic rejection) (3). Although the advancements in pre-transplant immune monitoring and immunosuppression regimens lead to decreased incidence of hyper acute and acute rejection, chronic rejection remains a major hurdle of long term graft function (4). Therefore, novel treatment strategies need to be developed to improve the outcome after kidney transplantation. In order to achieve this, it is crucial to understand the major and critical players beyond B and T cells. Since Natural Killer (NK) cells can influence T-cell- or antibody-mediated allograft rejection (5) and have a central role in anti-viral immunity (6, 7), it would be highly relevant to further explore their role in kidney transplantation.

NK cells are innate lymphocytes that represent 5-15% of lymphocytes in peripheral blood, they are derived from common lymphoid progenitors, they can mediate both cytotoxic and cytokine producing effector functions, and they have been recognized primarily for their contribution to the immune response against intercellular pathogens and malignant cells (8). However, increasing evidence suggests a role for NK cells in allograft rejection after kidney transplantation as well (9–11). In contrast to T cells, NK cells do not require priming with an antigen and NK cell activation is regulated by the balance between inhibitory- and activating receptors. Engagement of inhibitory NK cell receptors with their cognate ligands will trigger an inhibitory signalling cascade in the NK cells, hence, setting the threshold for activation of the NK cells (12). Activating NK cell receptors, on the other hand, typically interact with ligands that are associated with cellular stress or with viral infection and that are highly expressed on virus-infected or malignant cells (13–15). An excess amount of activating receptor-ligand interaction will trigger NK cell activation even in the presence of low levels of inhibitory signaling, a condition called "induced-self" (16). NK cells will normally not attack healthy cells, since they do not, or only very lowly, express activating ligands (16).

MHC class I molecules are the most important inhibitory ligands for NK cells. Under normal circumstances, recognition of self MHC molecules by the inhibitory receptors prevents NK cell activation against host cells and creates self-tolerance (17). However, virally infected- or malignant cells frequently downregulate MHC class I molecules to escape from T cells and this decreased expression of HLA molecules lowers the threshold for NK activation making only a very small activating signal enough to trigger NK effector responses, a condition that is also known as 'missing self' (18). Simultaneously, the expression of ligands for activating receptors increases due to cellular stress, infection or tumorigeneses, and a shift from inhibitory signals to activating signals results in activation of NK cells, leading to elimination of target cells *via* NK cell mediated cytotoxicity or through secretion of pro-inflammatory cytokines (19).

First evidence for the role of NK cells in rejecting allografts originates from murine studies showing that F1 hybrids can reject parental bone marrow cells upon transplantation, so called "hybrid resistance" (20, 21). In the late 1980's, the concept of NK cell "missing self recognition" was introduced after the observation that H-2 deficient lymphoma's were rejected in a NK cell dependent manner (22). Missing self recognition implies that, in the absence of engagement of inhibitory NK receptors with "self MHC class" molecules (or H-2 in mice), NK cells more readily respond to foreign- or malignant cells lacking expression of those self MHC molecules. Hence, providing a direct link between F1 hybrid resistance and missing self recognition, and an explanation for NK cell allorecognition in bone marrow transplants. Traditionally, hybrid resistance has not been linked to solid organ transplantation and the Snell's third law of transplantation states that 'Grafts from either inbred parent strain to the F1 hybrid succeed' (23). As will be discussed in more detail below, this idea was challenged by more recent studies in mouse models showing the participation of missing self-induced activated NK cells in cardiac allograft endothelial damage and vascular rejection, thereby, providing support for the existence of NK cell alloreactivity in solid organ transplantation when the endothelial cells are predominantly from donor origin (10, 24).

Activated NK cells could influence allograft rejection in multiple ways (**Figure 1**): One way is *via* their influence on the adaptive arm of the immune system through their crosstalk with dendritic cells (DCs) (**Figure 1C**). The underlying mechanism is that DCs induce NK cell activation *via* secretion of cytokines such as type I IFN, IL-12 or TNFα (25–28), and that, as a result of this activation NK cells release TNFα and IFN-ϒ, which further promotes DC maturation (29, 30). Since DCs play a key role in T cell activation, enhanced maturation of DCs and NK-derived IFN-ϒ promote T cell activation, expansion, and Th1 polarization (31, 32). Infiltration of $CD56^{pos}$ NK cells has been associated with poor outcome of kidney transplantation and interstitial fibrosis (33, 34). A more recent study, used flow cytometry to obtain more in depth profiles of NK cells revealing that $CD56^{bright}$ NK cell infiltrates were enhanced in biopsies from patients experiencing T cell mediated rejection (TCMR) while $CD56^{dim}$ NK cell infiltrates characterized biopsies from patients with antibody mediated rejection (AMR) (35). Since $CD56^{bright}$ NK cells are potent producers of cytokines, this was suggestive of a role for $CD56^{bright}$ NK cells in the recruitment and activation of alloreactive T cells (35). Moreover, this may be enhanced by the production of IFNϒ by activated T- or NK cells contributing to inflammation-induced enhanced expression of HLA alloantigens (35). Infiltration of $CD56^{dim}CD16^{pos}$ NK cells in AMR, would fit with the direct impact that NK cells can have on antibody-mediated allograft rejection *via* the Fc receptor CD16 expressed on the NK cell surface (**Figure 1B**) (36, 37). CD16, or FcϒRIIIa, can bind to the Fc part of donor specific antibodies (DSA), generated by the recipient B cells, that bind to the HLA or non-HLA molecules present on the allograft endothelial cell surface thereby inducing antibody dependent cellular cytotoxicity (ADCC) (38). In a mouse model for kidney transplantation, high DSA titres were paralleled with infiltration and proliferation of NK cells in the allograft, and in the absence of NK cells, the DSA could not provoke acute AMR and triggered progressive chronic kidney injury and the enhanced expression

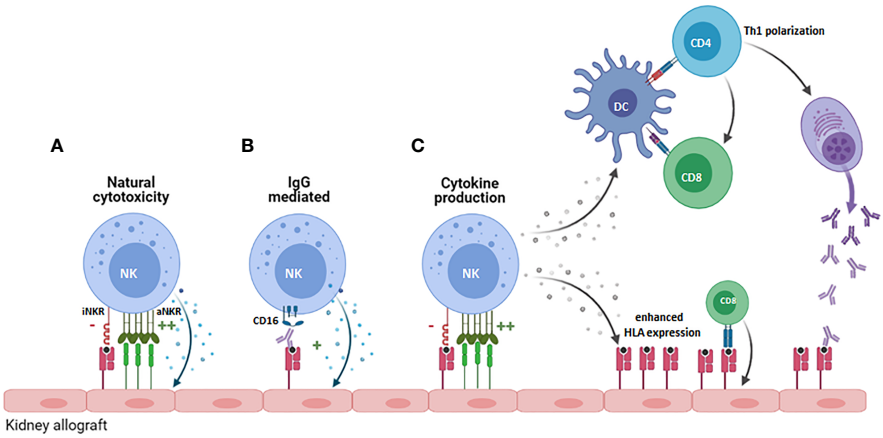


FIGURE 1 | Potential role for NK cells in promoting allograft rejection. NK cells can contribute to allograft rejection in several ways: **(A)** By mediating direct cytoxicity against cells of the allograft that increasingly express cellular-stress or virus-associated activating ligands. **(B)** *Via* antibody-dependent cellular cytoxicity upon binding of CD16 on the NK cell to anti-HLA antibodies. **(C)** By producing proinflammatory cytokines like IFN-ϒ, that promote Th1 polarization of $CD4^+$ cells, priming and activation of $CD8^+$ T cells directed against the allograft and by stimulating B cell production of pathogenic IgG antibodies. iNKR, inhibitory NK cell receptor; aNKR, activating NK cell receptor.

of pro-fibrotic genes leading to failure of kidney function (39). In humans, NK cells have been shown to contribute to chronic AMR by participating in DSA-induced microvascular inflammation (MVI) (33, 40). Consequently, NK cells can significantly contribute to the damaging effects that DSA can have on graft endothelial cells and by mediating DSA-induced ADCC they can also worsen the outcome of complement-independent chronic AMR (40).

In addition to producing cytokines and mediating ADCC, NK cells can directly damage donor endothelial cells that overexpress activating NK cell ligands (**Figure 1A**) or lack inhibitory HLA ligands for the patients NK cells as a result of mismatched HLA class I molecules between donor and recipient cells, a concept called "missing-self recognition" (10). The functional relevance of this type of response was elegantly demonstrated in a study showing chronic vascular rejection in approximately half of the DSA negative kidney transplant patients (10). In subsequent *in vitro* and *in vivo* models, missing-self by itself was not sufficient for the NK cells to damage graft endothelial cells. However, in combination with Poly-I:C (as surrogate for viral infection) or ischemia/reperfusion as provoking factor for NK cell activation and expression of activating ligands on endothelial cells, microvascular inflammatory lesions and allograft rejection occurred in an NK cell dependent manner (10). While chronic AMR is difficult to treat with existing immunosuppressive agents, mTOR inhibitors were effective in reducing NK-mediated rejection in this preclinical model (10), illustrating that better understanding of the role of NK cells in solid organ transplantation and how they synergize with the adaptive immune system could help to develop improved personalized immunosuppressive therapies.

The difficulty with studying NK cells in the kidney transplantation setting is that they act as a double edged sword. In addition to the above mentioned role in promoting acute- and chronic allograft rejection, a role for NK cells in inducing transplant tolerance has been proposed by several groups [reviewed in (41)]. One of the primary reasons is that NK cells in the stem cell transplantation setting have been shown to kill immature DCs and by doing so they prevent activation of alloreactive T cells and graft versus host disease (42). Comparable observations have been made in skin transplantation models (43). Moreover, NK cells have been shown to be able to kill highly activated T cells and to induce regulatory T cells which would also contribute to tolerance (41).

Besides allograft rejection, infection is an important contributor to the morbidity and mortality after kidney transplantation (44). Kidney transplant recipients receive immunosuppressive therapy in order to prevent rejection and to maintain allograft function, however, this therapy makes the transplant patients predisposed to various infections including viral infections. NK cells are critical players in the antiviral immune response and their contribution in controlling viral infections is a second reason why NK cell activation could be very beneficial in the transplant setting. The most frequent viral infections post transplantation occur mainly with viruses such as cytomegalovirus (CMV), Herpes simplex (HSV), Epstein Barr Virus (EBV), BK polyomavirus, Hepatitis B and C (44). These infections can drive from primary infection, transmission from the donor and reactivation of latent infections. Their effect on transplantation outcome emerges *via* different mechanisms; infection can either lead to an invasive disease such as CMV disease seen upon CMV reactivation in immunosuppressed individuals (45) or can contribute to graft rejection indirectly (46, 47). As a response to infection, virus specific CD8+ T cells can be formed, which can show cross reactivity with donor alloantigens (also known as 'molecular mimicry) inducing alloreactive response against donor cells and eventually leading to rejection (48). In a healthy individual, CD8+ T cells and NK

cells are primary responders and controllers of viral infection. Even more, NK cells have been reported to keep the CMV infection under control in the absence of CD8+ T cells in a child with severe combined immunodeficiency syndrome (49). Both CD8 T cells and NK cells kill virally infected cells by the release of granules containing perforins and granzymes leading to a reduction in membrane integrity and the activation of apoptosis promoting caspase activity and resulting in lysis of the target cell (50). Activation of CD8 T cells and NK cells is very different: CD8 effector T cells get activated upon interaction with a cell presenting viral peptides on MHC class I, while NK cells require the expression of virally-induced ligands for receptors like NKp30 and NKp46 (16). Since MHC class I acts as an inhibitory ligand for NK cells, NK cells are especially useful in eliminating target cells that reduced MHC class I in an attempt to escape from CD8 T cell immunity (17).

In kidney transplant recipients, it has been shown that reduced NK cell function correlates with and is a predictor of severe infection indicating the requirement of intact NK cell function in the defence against viruses (49, 51). Besides, the number of activating NK cell receptor genes of the Killer Immunoglobulin-like receptor (KIR) family in the recipients have been associated with decreased incidence of Human Cytomegalovirus (HCMV) *de novo* infection and reactivation in the first year of kidney transplantation (52). Moreover, the KIR gene repertoire can determine susceptibility of the patients to HCMV infections and the severity of the infection in renal transplant patients (53). Although the phenotypic and functional profile of NK cells has been reported to be altered under the immunosuppression treatment (54), their recovery has been detected to be faster than T cells (55). All these findings indicate the contribution and significance of NK cells in response to anti-viral defence, even in the absence of T cell response, in kidney transplantation.

The biology of NK cells, the multiple roles NK cells can have in organ transplantation and a detailed analysis of the interplay between viruses and NK cells have been excellently reviewed before, for example in (5, 41). For an overview on these topics we refer to these previous reviews. In the present review, we will primarily focus on the impact of HLA class I on NK cell alloreactivity and anti-viral immunity in the kidney transplantation setting and how this may impact transplantation outcome. This is an important topic because, although NK cells can respond to a plethora of ligands, classical and non-classical HLA class I molecules represent the most important inhibitory immune checkpoints for NK cells. As will be discussed in more detail in this review, the magnitude of the effect of HLA can depend on the degree of matching between donor and recipient and on the local microenvironment in the graft. Moreover, there are different models describing how the interaction of HLA molecules and NK cell receptors can affect the outcome in kidney transplantation. In the current review, we aim to describe those models and highlight recent findings regarding the HLA class I molecules as immune checkpoints in the regulation of NK cell alloreactivity in kidney transplantation as well as their influence on NK cell responses in viral disease occurring upon transplantation. In addition we will provide an overview of the state of the art methods used to identify NK cell receptors and HLA molecules and we discuss their limitations.

CLASSICAL HLA CLASS I MOLECULES AS LIGANDS FOR INHIBITORY- AND ACTIVATING NK CELL RECEPTORS

The highly polymorphic classical HLA class I molecules HLA-A, HLA-B and HLA-C are critical regulators of NK cell activation. These molecules are expressed by virtually every healthy cell and they present peptides derived from intracellular proteins on their cell surface and by doing so they play a critical role in discrimination between self- *vs* non-self- or diseased cells (56). Classical HLA class I molecules can interact with inhibitory- as well as activating NK cell receptors of the KIR family. Moreover, during NK cell functional maturation, their interaction with inhibitory KIR (iKIR) family members results in licensing of NK cells and licensed NK cells can more potently respond upon activation by a potential target cell than their unlicensed counterparts (19, 57). Licensing -or 'NK cell education'- is the process where NK cells interact *via* iKIR with classical HLA class I molecules expressed on for example stromal cells in the bone marrow, and licensed NK cells are characterized by an increased density in cytotoxic granules as well as a slightly altered metabolism with enhanced levels of glycolysis (58). To avoid excessive activation of NK cells against normal healthy cells, HLA class I molecules also act as potent inhibitory ligands and by doing so, they set the threshold for NK cell activation (59).

NK cells interact with classical HLA *via* so called KIR receptors, encoded on chromosome 19. The KIR family comprises several inhibitory- and activating family members and they are named by the presence of two (KIR2D) or three (KIR3D) extracellular immunoglobulin domains. Activating family members (aKIR) have a short (KIRxDSx) intracellular domain with immunoreceptor tyrosine-based activation motifs (ITAMs) and, although some of them recognize HLA class I, for several of the aKIRs the ligands remain elusive. Inhibitory family members have a long (KIRxDLx) intracellular domain with immunoreceptor tyrosine-based inhibitory motifs (ITIM) and most of them have HLA class I as ligands (60). Like their HLA ligands, KIRs are highly polymorphic and differences in expression levels due to copy number variation and allelic variation (including several known null-alleles) have been described (61). NK cells acquire KIR during maturation in a stochastic manner and can express none, one or a combination of KIRs, leading to high variation in expressed KIR repertoires between individuals as well as between NK cells within an individual (62). Moreover, depending on the exact set of *KIR*-genes, multiple haplotypes are known (https://www.ebi.ac.uk/ipd/kir/sequenced_haplotypes.html). The so called framework KIRs (*KIR3DL3, KIR3DP1, KIR2DL4* and *KIR3DL2*) are present in every haplotype (63). Furthermore, the haplotypes can be distributed in haplotype A or haplotype B (**Figure 2A**). The A haplotype consists of *KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DL2, KIR3DL3, KIR2DP1* and

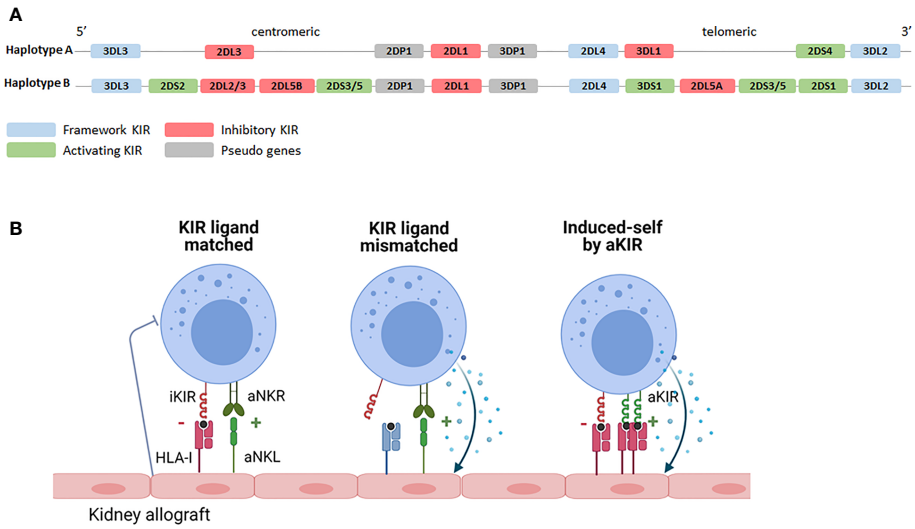


FIGURE 2 | KIR haplotypes and the potential effects of KIR-HLA interaction. **(A)** Based on the *KIR* gene content two haplotypes can be distinguished. The A haplotype containing *KIR2DS4* as the only activating receptor and the B haplotype containing multiple combinations of activating- and inhibitory *KIR* genes. Depending on the exact combination of *KIR* genes, multiple different B haplotypes are known (https://www.ebi.ac.uk/ipd/kir/sequenced_haplotypes.html). **(B)** Recipient NK cells may encounter their HLA class I ligands on the kidney allograft (KIR ligand match) or not (KIR ligand mismatch). Even in the presence of class I ligand, stress- or infection associated ligands for activating receptors, including activating KIR, on the allograft can trigger NK cell cytotoxicity (induced-self by aKIR). iKIR, inhibitory killer immunoglobulin-like receptor; aKIR, activating killer immunoglobulin-like receptor.

KIR3DP1 and only *KIR2DS4* as activating receptor (63). The set of *KIR* genes present in the B haplotype is much more diverse and multiple aKIRs are typically expressed (63). As will be discussed in more detail later, the introduction of next generation sequencing (NGS) led to a continuous increase in the number of *KIR* alleles and haplotypes.

IMPACT OF KIR-HLA CLASS I INTERACTION ON KIDNEY TRANSPLANTATION OUTCOME

KIR-ligand interaction may influence transplantation outcome on the one hand by promoting NK cell activation through the interaction between activating family members and their HLA ligands or by dampening NK cell activation *via* inhibitory family members. The influence of iKIRs is best described for KIR2DL1, binding to HLA-C alleles harboring the C2 epitope (lysine at AA position 80); for KIR2DL2/3 that predominantly interact with HLA-C alleles with the C1 epitope (asparagine at AA position 80); and for KIR3DL1 binding to HLA-B alleles with a Bw4 epitope and to HLA-A*23/*24/*32 (64, 65). Since many kidney transplantations are performed with one or more mismatches in HLA-A, -B or -C, iKIR receptors expressed on the patients NK cells may or may not encounter their HLA class I ligands on cells of the transplanted kidney, a so called "KIR-ligand match" or "KIR-ligand mismatch" (**Figure 2B**). The functional relevance of a KIR-ligand mismatch is that the amount of inhibitory signals provided to the NK cells is reduced making the NK cells more prone to attack a potential target cell. In a landmark paper by Ruggeri et al., patients that received a haploidentical stem cell graft that was iKIR-ligand mismatched in the graft *vs.* host direction had less relapse of disease which could be attributed to a better anti-tumor response of graft NK cells (42). In addition, these patients developed less graft-versus-host disease than patients receiving a KIR-ligand matched stem cell graft which could be explained by the enhanced killing of host immature dendritic cells by graft NK cells in iKIR-ligand mismatched receivers (42). Given the relatively short lifespan of NK cells and because of the lack of massive clonal expansion by NK cells, stable long term presence of donor NK cells seems rather unlikely in the kidney transplantation setting. Nevertheless, the presence of passenger donor NK cells, transferred from the donor to the recipient in the kidney allograft, has been shown at the time of transplantation (66). These donor NK cells have been suggested to get activated upon ischemia/reperfusion damage and *in vitro* studies showed that these activated NK cells can subsequently promote maturation of DC hence contributing to enhanced allorecognition by T cells (66). The role of recipient NK cells has been studied much more extensively and will be discussed in the following paragraph in more detail.

In kidney transplantation, recipient NK cells can contribute to graft rejection or antiviral immunity by mediating natural cytotoxicity, triggered upon encountering a target cell expressing high levels of stress- or infection- associated ligands for activating receptors (**Figures 1A**, **2B**). Alternatively they can contribute to AMR after engagement of the CD16 Fc receptor on the NK cell and an antibody bound to the cells of

the allograft (**Figure 1B**). Furthermore, upon activation, they can produce IFN-ϒ and by doing so act as adjuvants for the adaptive immune system by promoting Th1 and $CD8^+$ T cell activation (**Figure 1C**). iKIR licensed NK cells have been shown to have higher density granules than non-licensed cells enabling them to more potently mediate all these effector functions (58). Simultaneously, NK cells expressing iKIRs that encounter their cognate HLA ligand on the allograft will have a higher activation threshold than mismatched NK cells that do not meet their HLA ligand, making it relevant to address KIR- and HLA status in the kidney transplantation setting. The impact of KIR-ligand matching *vs* mismatching in HvG direction on graft rejection and/or graft survival has been addressed in multiple studies (see **Table 1**). In a cohort of 69 patients, Kreijveld et al. observed no association between the occurrence of acute rejection after reduction of immune suppression and KIR-ligand matching status, presence of KIR-ligands in the donor or NK cell frequencies or subsets (67). In line with those data, no impact of KIR-ligand matching status on long term allograft function was found in cohorts of 126 (68), or 2757 (69) renal transplant patients, the first one with 5 years follow up, the latter with 10 years. In another study, patients with stable renal function (n=119) were compared with patients experiencing acute rejection within the first 3 postoperative months (n=105), demonstrating that HLA-C ligand compatibility by itself had no influence on transplantation outcome while a higher number of inhibitory recipient *KIR* genes, a higher number of KIR2DL2/DS2 matches and a higher number of mismatches for KIR2DL3 were detected in the non-rejectors (70). Higher number of inhibiting KIR genes encountering their ligand might hinder NK cell activation and thus lead to less damage to the kidney. Indeed this is in accordance with the results of the study in which the absence of recipient KIR2DL1-donor HLA-C2 and/or recipient KIR3DL1-donor-HLA-Bw4 was significantly higher in patients experiencing chronic rejection (71), with the background that these combinations have higher NK inhibitory capacity than the combination KIR2DL2/3 with HLA-C1. In two studies (72, 73) the authors investigated the effect of KIR-ligand mismatches on long term graft survival in a cohort of patients transplanted with a HLA-A, -B, -DR compatible donor, with the idea that in this cohort the NK cell effects cannot be obscured by allo-reactive T and/or B cells. Indeed, in one study with 137 patients with HLA-A, -B, -DR compatible donors, KIR-ligand mismatches were associated with a 25% reduction in 10 year graft survival, whereas no effect was seen in 260 patients with a HLA-A, -B incompatible, HLA-DR compatible transplantation (72). However, in the other study no effect of KIR-ligand mismatches was observed in 608 patients with a HLA-A, -B, -DR compatible transplantation (73). These contradictory results were rather striking, because the groups seemed to be comparable. Whether this contradiction is due to the difference in numbers of patients is unclear. In one paper only deceased donors were included with HLA-A, -B and -DRB1 zero mismatch with the patient (73), whereas in the other paper both deceased and living donors were included that were HLA-A, -B, -DR compatible, which might have included donors matched for HLA-A and –B at the broad level instead of at the split level (72). Interestingly, the difference in graft survival between KIR-ligand match and mismatch appeared after 5 years posttransplantation, so no difference was observed in 5 years survival curves (72). Koenig et al. challenged the idea that antibodies are the only primary trigger for microvascular inflammation in kidney transplantation (10). In a cohort of 129 patients without DSA, and in *in vitro* and *in vivo* models, they showed that in approximately half of patients MVI lesions were not mediated by antibodies but by activation of innate immune cells and that the presence of a KIR-ligand mismatch in HvG direction enhanced this process (10). In a mouse model they also demonstrated that NK cell activation in response to KIR-ligand mismatched microvascular endothelial cells was mTORC1 dependent and inhibition of mTORC1 with rapamycin could prevent this type of rejection (10). In a recent follow up study with 1682 kidney transplant patients, the same authors demonstrated that KIR-ligand mismatches have an increased detrimental effect on transplantation outcome in patients with non-complement fixing DSA as they promote AMR mediated by DSA that trigger NK cell reactivity against graft endothelial cells (40). Also these two studies support the hypothesis that KIR-ligand incompatibility has a detrimental effect on allograft survival even in a situation with DSA.

Evaluation of the impact of KIR-ligand matching status, and comparison between clinical studies is complicated by multiple factors: First of all the differences in study design and clinical parameters like the exact transplantation protocol, the presence or absence of preexisting or *de novo* DSA, the outcome parameters used for evaluation and immunosuppressive regimens maybe different between studies. A second important factor is the definition of KIR-ligand incompatibility. Ideally the phenotypic presence of KIR receptors should be confirmed. This is important because the genes encoding iKIRs are not always present in every individual and for e.g. KIR2DL1 and KIR3DL1 null alleles exist (https://www.ebi.ac.uk/ipd/kir). When assessing the presence *vs* absence of the HLA ligands it is especially for Bw4 important to also consider the HLA-A alleles as HLA-A*23, -*24 and -*32 encode for the binding site for KIR3DL1 and this is not always consistently done between studies. Since NK cell can express one or more KIRs, and because there is a large inter-individual variation, the size of the alloreactive NK cell population (i.e. the size of the population of NK cells expressing only mismatched KIRs) can be very different on a per individual basis (62). Especially in smaller studies this may influence the overall impact of KIR-ligand mismatching. Thirdly, the microenvironment in the graft or the systemic inflammatory status of the patient may influence the impact of KIR-ligand incompatibility. Under homeostatic conditions, even in the situation with a complete lack of inhibitory signaling *via* HLA and KIR, unactivated NK cells will not attack healthy cells. The absence of inhibitory signaling is not enough to activate the NK cell and NK activation requires expression of activating ligands on a potential target cell (14). Viruses frequently encode activating NK ligands, and cellular stress or proinflammatory cytokines promote activation of NK cells by enhancing

TABLE 1 | Overview of published studies investigating the impact of KIR-ligand matching and mismatching on graft survival and graft rejection after kidney transplantation.

Patient Group	n	Cases	n	Controls	n	Outcome/Variable	Observations	Reference
reduced immune suppression	69	acute rejection	24	no acute rejection	45	peripheral blood NK cell frequency	no differences between case and control	Kreijveld et al. (67)
						presence of single KIR genes in recipient	no differences between case and control	
						presence of KIR haplotypes in recipient	no differences between case and control	
						presence of NK cell alloreactivity based on missing self	no differences between case and control	
						presence of NK cell alloreactivity based on missing ligand	no differences between case and control	
deceased donors	126	WGF (5year eGFR/ creatinine)	59	SGF (5 year eGFR/ creatinine)	67	NK alloreactivity (recipient KIR/HLA donor mismatch ligand)	no differences between case and control	La Manna et al. (68)
deceases donors	2757	KIR ligand incompatible	871	C1/2-Bw4matched	1416	graft survivalrate10 year follow up	no differences between case and control	Tran et al. (69)
		KIR ligand incompatible	871	C1/2-Bw4mismatched	470	graft survivalrate10 year follow up	no differences between case and control	
unrelated donors	224	with AR within 3months	105	With stable renal function	119	HLA·C ligand incompatibility	no differences between case and control	Kunert et al. (70)
						Donors homozygous for C2	higher in controls compared to cases	
						number of recipient inhibitory receptors	higher in controls compared to cases	
						number of donor ligand matches for recipient KIR2Dl2/DS2	higher in controls compared to cases	
						number of donor ligand mismatches for recipient KIR2Dl3	higher in controls compared to cases	
HLA-DR matched deceased donors	174	chronic rejection	42	SGF	132	Donors homozygous for C1	higher incases compared to control	Littera et al. (71)
						absence of rKIR2Dl1/dHLA-C2	higher incases compared to controls	
						absence of rKIR3DL1/dHLA-Bw4	higher incases compared to controls	
HLA·AB incompatible, DR compatible donors	260	KIR ligand mismatched	134	KIR ligand matched	126	10year graft survival	no differences between case and control	Van Bergen et al. (72)
HLA ABDR compatible donors	137	KIR ligand mismatched	42	KIR ligand matched	95	10year graft survival	25% reduction in graft survival cases of controls	
HLA-ABDR compatible, deceased donors	608	KIR ligand mismatched	193	KIR ligand matched	415	10year graft survival	no differences between case and control	Tran et al. (73)
Kidney transplant patients	760	C2present	457	C2 absent	303	long term graft survival	shorter in cases compared to controls	Hanvesakul et al. (66)
						acute rejection	no significant differences between case and control	
Kidney allograft biopsies: MVI+DSA+, non-complement	62	missing-self present	21	no missing-self present	23	graft survival	lower incases compared to controls	Koenig et al. (40)
Kidney allograft biopsies: MVI+, DSA+, complement	73	missing-self present	23	no missing-self present	17	graft survival	no difference between cases and controls	

WGF, worse graft function; SGF, stable graft function; AR, acute rejection.

expression of activating ligands on potential target cells or by increasing expression of activating receptors on NK cells (15). This makes viral status and factors like ischemia/reperfusion important influencers of the NK cell response in kidney transplantation.

NK cells are critical controllers of viral infections, this may be especially relevant in conditions were T cell mediated control is reduced as a result of immunosuppression regimes. HCMV infection is one of the most frequently occurring complications after kidney transplantation, a risk factor for the rate of graft loss and associated with reduced survival (74). Several studies confirmed the functional relevance of the *KIR* gene repertoire for HCMV infection upon kidney transplantation (see **Table 2**). Most pronounced are the associations observed for the presence of KIR haplotype B/X, especially the telomeric B haplotype, and reduced HCMV infection or reactivation (52, 75–77, 82), which presumably could be explained by the presence of a higher number of activating KIR's in the KIR B/X-haplotype as compared to the KIR AA-haplotype, facilitating NK cell mediated killing of virally infected cells. One study, however, describes the opposite effect in two independent cohorts, but both consisting of patients that were HCMV negative at the time of transplantation, but transplanted with an HCMV positive donor (53). In both cohorts the KIR telomeric haplotype B/X in combination with HLA-C2 was significantly associated with susceptibility to HCMV infection, whereas the KIR haplotype AA in combination with HLA-C1 was protective for development of severe disease (53). In another study the significant effect of the KIR B haplotype as protection for HCMV infection, was only detected in patients that were already HCMV seropositive at the time of transplantation (77). A third study with 90 kidney patients that were HCMV negative and transplanted with a HCMV positive donor showed a trend towards a lower incidence of HCMV infection in recipients with KIR AA haplotype (81). It is not clear which biological mechanism is underlying these findings for HCMV seronegative patients at the time of transplantation and whether the lack of T or NK cell memory in this specific group in combination with immunosuppression of T cell responses during the initial stage of infection plays a role. A protective role for KIR B haplotypes has also been observed for Varicella zoster (78) and BK virus (79) though two other studies did not find a significant impact of KIR haplotypes on BK virus (78, 80). The impact of KIR repertoires on other viral infections is not very well studied.

The relevance of inhibitory KIRs and of KIR-ligand incompatibility on control of viral infections is less conclusive and less well explored. In a first study, the absence of HLA-C ligands for the recipients inhibitory KIRs associated with reduced HCMV infection rate after transplantation (75). While a second study did not observe differences in *KIR* gene and genotype distribution and no effect of KIR-ligand mismatching for patients with or without HCMV (81). Also individual KIRs have been related to HCMV infection. In a study with 138 kidney transplantation recipients, the lack of KIR2DS2, the presence of KIR2DL3 or the combination of KIR2DL2 and HLA-C1 were identified as risk factors for HCMV infection (82). Moreover, enhanced numbers of KIR3DL1 positive NK cells have been observed early upon HCMV reactivation, and in an *in vitro* study, the authors subsequently demonstrate that this subset most efficiently kills HCMV infected fibroblasts (83).

The multiple studies showing associations between KIR haplotypes and kidney transplantation outcome illustrate the relevance of further exploring the functional consequences of KIR repertoires in combination with analysis of KIR ligands, in kidney transplantation outcome. In the following paragraphs we will discuss the different methods that can be used to determine KIR and HLA genotypes enabling such future studies.

METHODS TO DETERMINE THE PRESENCE OF HLA CLASS I KIR LIGANDS

KIR ligands are defined by single amino acid differences on HLA-B and -C molecules. The HLA-B molecules can be divided into two supertypic specificities, Bw4 and Bw6, with amino acid differences at positions 77 and 80-83 of the mature protein. Bw6 is defined by serine at residue 77 and asparagine at residue 80, whereas Bw4 is characterized by at least seven different patterns of amino acids at positions 77 and 80-83. Complicating factor is that the Bw4 motif is also present on several HLA-A molecules. HLA-B and HLA-A (A*23, *24, *32) alleles carrying the HLA-Bw4 epitopes are recognized by KIR3DL1, the Bw6 epitope is not a ligand for KIRs. The HLA-C molecules carry either a C1 or a C2 motif, based on a dimorphism at residue 80. The C1 motif is defined by the presence of an asparagine at position 80, whereas the C2 motif has a lysine at position 80 of the mature protein (84).

To determine these amino acid differences several different methods are available. HLA typing has started with serological determination, discriminating HLA molecules by incubating cells with known HLA antibodies using the complement dependent cytotoxicity (CDC) method. Since Bw4 and Bw6 are potent public antibody epitopes, specific antibodies against these supratypes are present and can be used to distinguish these motifs. However, nowadays HLA typing is merely determined by molecular typing methods using genomic DNA. Due to the high polymorphism a special HLA allele nomenclature has been developed for molecular HLA typing results, an example and explanation of this nomenclature is depicted in **Figure 3**. Molecular HLA typing can be performed at low or high resolution level, determining respectively the allele groups (comparable to serological types) or the alleles present, illustrated in **Figure 4**. Although most alleles within an allele group bear the same NK ligand motif, there are some exceptions as described previously (87) and as can be deduced from the protein sequences of the alleles available at the IPD-IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla) (85). Therefore, low resolution HLA typing will not be sufficient to determine the NK ligands with certainty. In contrast, high resolution typing will determine the protein sequences of the peptide binding

TABLE 2 | Overview of published studies investigating the impact of KIR and KRI-ligand on viral infections after solid organ transplantation.

Patient Group	n	Cases	n	Controls	n	Outcome/Variable	Observation	Reference
kidney transplant patients	122	KIR haplotype B/X	82	KIR haplotype AA	40	rate of CMV infection 1st year after tx	significantly lower in cases (20%) compared to controls (36%)	Stern et al. (52)
						graft function	no significant differences between case and control	
						rate rejection episodes	no significant differences between case and control	
						rate of EBV, BKV, Herpes simplex	no significant differences between case and control	
kidney cohort 1:HCMVD+R- patients with antiviral prophylaxis	76	>500 copies HCMV/ml within first 6months	24	<500 copies HCMV/ml	52	frequency of Tel B KIR genes	higher in cases compared to controls	Jones et al. (53)
						Freq Tel B +HIA-C2	higher in cases compared to controls	
kidney cohort 2: HCMV D+R- patients without antiviral prophylaxis	65	HCMV >50 infected cells	12	HCMV<10 infected cells	35	Tel AA haplotype	significantly lower incases compared to controls	
						Tel A/X +HIA-C1	significantly lower in cases compared to controls	
						Tel B/X haplotype	significantly higher in cases compared to controls	
						Tel B/X +HLA-C2	significantly higher in cases compared to controls	
						Homozygous HLA-C2	significantly higher in cases compared to controls	
kidney transplant patients	196	two missing KIR ligands	38	no or 1 missing KIR ligand	158	rate of CMV infection up to 3 months	significantly lower in cases compared to controls	Hadaya et al. (75)
		HLA-C missing KIR ligand	103	no HLA-C missing KIR ligand	93	rate of CMV infection up to 3 months	significantly lower in cases compared to controls	
		patients with more activating KIR genes		patients with fewer activating KIR genes		rate of CMV infection up to 12 months	each additional activating KIR gene reduced risk of CMV event by 19%	
kidney transplant patients	339	patients with KIR Cen BX haplotype	192	patients with KIR Cen AA haplotype	147	rate of CMV infection up to 12 months	no significant differences between case and control	Stern et al. (76)
		patients with KIR Tel BX haplotype	158	patients with KIR Tel AA haplotype	181	rate of CMV infection up to 12 months	significantly lower in cases compared to controls	
kidney patients excluding D-R-	223	patients with KIR B/X haplotype		patients with KIR AA haplotype		cumulative incidence of CMV in first 2 years	no significant differences between case and control	Gonzalez et al. (77)
kidney patients excluding D-R, receiving ATG	40	patients wtih KIR B/X haplotype		patients with KIR AA haplotype		cumulative incidence of CMV in first year	38% incases *vs* 48% in controls	
heart, kidney, liver, lung tx patients	649	patients with KIR B/X haplotype	473	patients with KIR AA haplotype	176	cumulative incidence of varicella zoster infection (n=28)	significantly lower in cases compared to controls	Schmied et al. (78)
		patients with KIR B/X haplotype	473	patients with KIR AA haplotype	176	Cumulative incidence of EBV, HSV, BKPyV	no significant differences between case and control	
kidney transplant patients	158	patients with severe BKV reactivation	48	patients with no BKV and stable function first 6 years after transplant	110	Tel B/X haplotype	significantly lower incases compared to controls	Trydzenskaya et al. (79)
						Low number of activating KIR genes (<4)	significantly higher percentage incases compared to controls	
						presence of KRI3DSI	significantly higher in controls compared to cases	
						KIR/HLA match and mismatch	no significant differences between case and control	
kidney transplant patients	103	patients with KIR B/X haplotype	75	patients with KIR AA haplotype	28	cumulative incidence of BK virus in first 2 years	no significant differences between case and control	Brochot et al. (80)

(Continued)

TABLE 2 | Continued

Patient Group	n	Cases	n	Controls	n	Outcome/Variable	Observation	Reference
kidney transplant patients D+R-	90	patients with KIR B/X haplotype	63	patients with KIR AA haplotype	27	cumulative incidence of CMV in first year	trend towards lower incidence in controls (30%) *vs* cases (48%)	Michelo et al. (81)
		one or more missing KIR ligands	38	no missing KIR ligand	52	cumulative incidence of CMV in first year	no significant differences between case and control	
kidney transplant patients	138	patients with KIR B/X haplotype	96	patients with KIR AA haplotype	42	cumulative incidence of CMV in first 2 years	trend toward slower incidence in cases (31.2%) *vs* controls (47.6%)	Deborska-Materkowska et al. (82)
		CMV infection	50	no CMV infection	88	lack of KIR2DS2	significantly higher in cases compared to controls	
		CMV infection	50	no CMV infection	88	presence of KIR2DL3	significantly higher in cases compared to controls	
		CMV infection	50	no CMV infection	88	presence of KRI2DL2-HLA-C1	significantly higher in cases compared to controls	

D+, HCMV positive donor; R-, HCMV negative recipient; D-, HCMV negative donor; MVI, microvascular inflammation; DSA, donor specific antibodies.

groove, encoded by exons 2 and 3 of the HLA class I gene, as a minimum, which is sufficient for defining the NK binding region.

For high resolution typing, sequencing is the most reliable method, because then the complete nucleotide sequence of at least exons 2 and 3 is determined. Other methods are PCR-SSP, based on amplification of genomic DNA with sequence specific primers analyzed by gel electrophoresis, and PCR-SSO, based on amplification of the locus of interest followed by hybridization with sequence specific oligonucleotide probes. Although typing can be performed with these methods, the problem exists that not all nucleotides are determined, only the ones for which polymorphism has been identified in the past. However, for determination of NK ligand positions all of these methods can be used.

Determining the nucleotide sequences can be done with either Sanger sequencing or with next generation sequencing. For both methods the HLA gene of interest is amplified and this amplification comprises at least exons 2 and 3, but can be extended to the full length gene. Subsequent sequencing by the Sanger method might result in an ambiguous allele assignment, because the two alleles were not separated before sequencing and therefore, the cis-trans positions of nucleotides cannot be determined. To circumvent this problem, an allele-group specific amplification can be performed as described previously (88). Alternatively, next generation sequencing also separates the alleles in their processing steps, enabling unambiguous allele assignment as well. Different next generation sequencing methods for HLA class I (HLA-A, -B, -C) have been described, varying from second generation (Illumina, Ion Torrent, Mia Fora), in which small overlapping fragments are sequenced (89–96) to third generation (PacBio), in which a single molecule template is used for sequencing (86, 97), with the notification that for the second generation NGS cis-trans positioning can still be a problem if polymorphism in overlapping fragments is not sufficiently high, resulting in phasing problems and an ambiguous allele assignment. The latest development, often called the fourth generation (Nanopore MinION) is also based on a single molecule, but no nucleotide incorporation is needed, because the nucleotides are identified by passing an electric signal through a nanopore. This latter method is now also validated and implemented for HLA class I high resolution typing (98). By typing to the allele level the KIR ligand epitope is defined.

Since sequencing used to be an expensive and labor intensive method and since NK ligand binding of HLA class I is limited to the amino acid region 77-83, several other methods, focusing only on the polymorphism defining the KIR ligand specificity, were developed in the past. Among them are: (1) a SNP (single nucleotide polymorphism) assay (99), amplifying part of the gene and using fluorescent probes distinguishing between C1 and C2 and between Bw4 and Bw6, the latter one being based on the nucleotide dimorphism at position 319 (amino acid 83), (2) a qPCR Taqman method (100), using HLA-C group 1 and group 2 specific amplification primers in combination with a generic probe for quantification, (3) a PCR-SSP method (101) using sequence specific primers for HLA-C group 1 and group 2

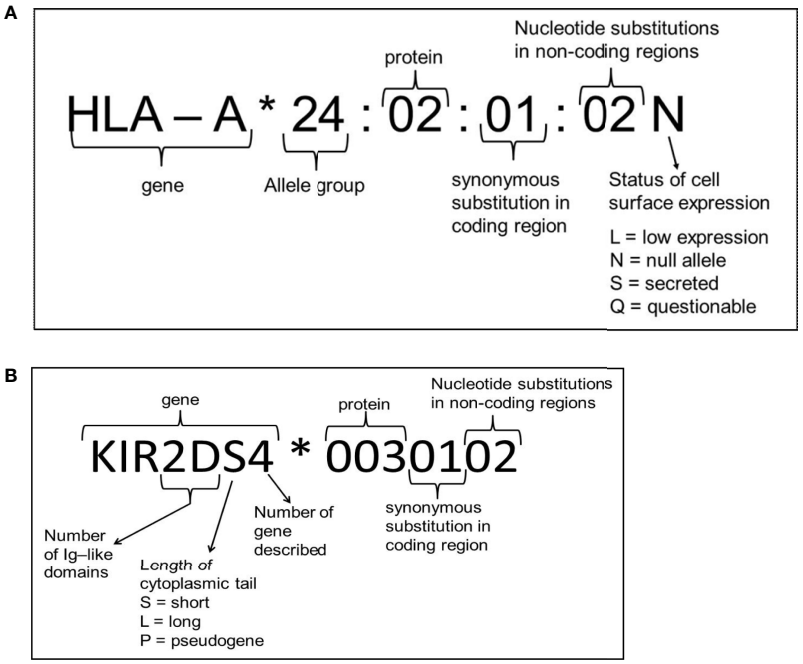


FIGURE 3 | Explanation of nomenclature used for HLA **(A)** and KIR **(B)**. Figure adapted from the IPD-IMGT/HLA and IPD-KIR database website, respectively (85, 86).

analyzed by gel electrophoresis and (4) a pyrosequencing method (102, 103) based on direct sequencing of the actual ligand epitope and detection by release of pyrophosphate. Furthermore, although the Luminex bead based SSO method, using sequence specific probes bound to fluorescently labeled beads, generates a low resolution typing, it is still possible to determine the HLA ligand presence, because there are probes on the beads that specifically can bind the NK ligand region, both for HLA-B as well as HLA-C. Positivity of these beads implicates that the NK ligand is present.

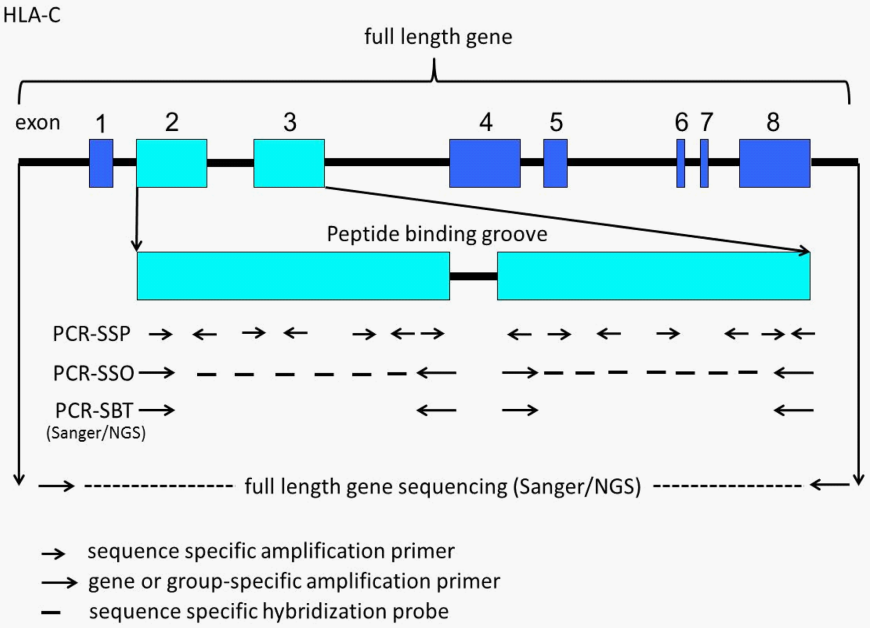
However, all these methods, that only identify the crucial positions for KIR binding, are not able to detect if the allele present will be really expressed or not. Among both HLA-B and -C (as well as -A) there are now many alleles identified that are not expressed, the so called null alleles. Although their frequencies in the population are very low (104), the impact on NK alloreactivity is huge. Therefore, high resolution typing of HLA-A, -B and -C, according to the standards of the European Federation for Immunogenetics (EFI) taking null alleles into account, would be the definite correct way of determining NK ligand presence.

METHODS TO DETERMINE KIR

The KIR genes are comparable to HLA, they also show a high degree of polymorphism, correlated with population groups of specific geographic ancestry, and the more they are studied, the more different alleles are identified (105, 106). However, KIR has two extra layers of variability compared to HLA in that genes can be present or absent and the copy number of genes can vary (107–110). Also for KIR typing different methods are available: methods to determine which genes are present, methods to define how many copies are present and methods to identify the alleles.

Most methods that exist for KIR typing focus on the gene content. Determination of KIR genes present can be performed by PCR-SSO, preferably with Luminex technology so that many samples can be analyzed simultaneously (111), (multiplex) PCR-SSP (112–115), real time PCR (116) or multiplex PCR followed by NGS (117). With these KIR genotyping methods it is also possible to determine the presence of KIR haplotypes A and B, based on the KIR gene presence. The KIR haplotype A has a fixed number of 7 genes (2DL1, 2DL2/3, 2DL4, 2DS4, 3DL1, 3DL2, 3DL3) and 2 pseudogenes (KIR2DP1, KIR3DP1), whereas the KIR haplotypes B vary in KIR gene content, including at least one of the following genes KIR2DL5, 2DS1, 2DS2, 2DS3, 2DS5 and 3DS1 (106, 108, 109, 118).

For defining the number of copies (CNV = copy number of variation) of the different KIR genes different methods have been used, among them quantitative PCR (119), droplet digital PCR (120), multiplex ligation dependent probe amplification (MLPA) (121) and NGS (105, 122). For identifying the KIR alleles, sequencing of the KIR gene is needed. To our knowledge this has only been performed by NGS methodology (105, 122, 123). The Stanford University has developed a method to determine all in one, that means HLA class I KIR ligand typing together with KIR gene presence, copy number and allelic typing. This is achieved by capturing the KIR and HLA class I genes by



method	region analyzed	resolution	example of HLA typing
serology		low	A24
PCR-SSP	exons 2 and 3	low	A*24
PCR-SSP	exons 2 and 3	intermediate	A*24AGK
PCR-SSO	exons 2 and 3	low	A*24
PCR-SSO	exons 2 and 3	intermediate	A*24AGK
PCR-SBT (Sanger/NGS)	exons 2 and 3	intermediate	A*24:02G
PCR-SBT (Sanger/NGS)	exons 2 + 3 + null alleles	high	A*24:02P
full length sequencing (Sanger/NGS)	full length gene	allelic	A*24:02:01:01

FIGURE 4 | Illustration and comparison of different HLA typing methods and the generally obtained typing resolution level. The figure depicts HLA-C as an example. Depending on the number of sequence specific primers or probes the PCR-SSP/SSO method can have low or intermediate resolution typing result. A*24AGK: string of different A*24 alleles. A*24:02G: group of alleles with identical peptide binding groove, but differences outside (including null alleles). A*24:02P: group of proteins with identical peptide binding groove, but differences outside (excluding null alleles).

specific probes, followed by Next Generation Sequencing and a sophisticated bioinformatics pipeline for analysis (105).

As for HLA there is also a database for KIR alleles (https://www.ebi.ac.uk/ipd/kir/) (124) with rules for the assignment and nomenclature of alleles comparable to HLA (125) (**Figure 3**). At present there are 1532 KIR alleles identified, according to this database (vs2.10.0), encoding 668 different proteins, whereas 17 null alleles have been found.

NON-CLASSICAL HLA CLASS I MOLECULES AS LIGANDS FOR NK CELL RECEPTORS IN KIDNEY TRANSPLANTATION

In contrast to the highly polymorphic classical HLA class I molecules, non-classical HLA class I molecules display a

relatively limited polymorphism. HLA-E and HLA-G are the most frequently studied ligands for NK cells and especially their immunosuppressive effects through inhibitory receptors are well characterized. In the following paragraphs the potential impact of the interaction between non classical HLA class I molecules and NK cells expressing receptors that can interact with these molecules on kidney transplantation will be discussed.

HLA-E as Ligand for NK Cells Expressing NKG2A and/or NKG2C

HLA-E has a gene- and protein structure that is highly comparable to the classical class I molecules but only two main protein variants are known HLA-E*01:01 and HLA-E*01:03 (126, 127). HLA-E can inhibit NK cells and T cells and has been shown to induce regulatory T cells and as a result expression of HLA-E in tissues has been associated with immune suppression in pregnancy and cancer and with viral immune-escape (128). For cell surface expression, binding of a conserved nonamer peptide is required. Due to a single amino acid difference (R107G), the HLA-E*01:03 variant has a higher peptide binding affinity resulting in a higher level of expression on the cell surface (126, 127). Both variants present peptides derived from the leader sequences of HLA-A, -B or -C as well as peptides from viral- and stress proteins (e.g. CMV or Hsp60) (129, 130). The role of this functional dimorphism of HLA-E in solid organ transplantation has been studied in only a very limited number of studies. One study showed a protective role of the HLA-E*01:01/01:01 genotype of the donor for rejection in 107 kidney transplantation donor-recipient pairs (131). In another study, living-donor kidney recipients with HLA-E*01:01/01:01 experienced less BK polyoma virus (BKPyV) reactivation than recipients with other genotypes, and BKPyV-induced nephropathy occurred more frequently in recipients carrying the HLA-E*01:03 allele (132). In both studies, direct effects on NK cell reactivity were not addressed and larger studies are required to draw strong conclusions on the impact of the HLA-E functional dimorphism.

HLA-E can interact with the TCR on CD8 and regulatory T cells and with receptors of the lectin-like family like NKG2A and NKG2C that are expressed on NK cells and some T cell subsets (133). This clearly illustrates that HLA-E can provoke both immune activating as well as inhibitory effects (133). While both NKG2A and NKG2C heterodimerize with CD94, the inhibitory NKG2A family member binds HLA-E with a higher affinity than the activating NKG2C receptor (134). For both receptors, the outcome of receptor ligand interaction depends primarily on the HLA-E expression level as well as on the exact peptide presented in HLA-E (135). Presentation of cellular stress-associated Hsp60 peptides for example does not lead to inhibition of NK cell effector function, while presentation of HLA class I leader peptides or peptides from viral origin does (136, 137). For NKG2C, interaction with HLA-E complexed to an HLA-G derived peptide most potently stimulates NK cells (136, 138). Like the inhibitory KIR family members, NKG2A is involved in licensing of NK cells and NKG2A licensed NK cells can mediate more potent responses against HLA-E negative target cells than their non-licensed hyporesponsive counterparts that do not express KIR or NKG2A (139). Together with the iKIRs, NKG2A is critical in maintaining NK cell tolerance for healthy cells (133). Expression of NKG2C, on the other hand, is largely influenced by viral status of the patients and has been associated with improved anti-viral NK cell responses and long-lived NK cells that have acquired features resembling the adaptive immune response (140, 141).

The functional impact and imprinting that viruses can have on the NK cell compartment is most clearly illustrated by CMV. For a more complete overview on all the studies addressing this topic, we refer to (142), here we will only briefly discuss the impact of CMV on the NK cell receptors interacting with HLA class I because expression levels of both NKG2A and NKG2C are heavily influenced by CMV. One of the first studies providing evidence for this showed that in CMV seronegative individuals, the percentage of NKG2A+ NK cells ranged between 23.5-62.7% while this was between 12.3-75% in CMV positive individuals (143). In the same study, the percentage of NKG2C+ NK cells ranged between 0.1-6% in CMV negative individuals and between 2.5-80% in CMV positive individuals, clearly illustrating the impact of viral imprinting on the NK cell repertoire. Lopez et al. showed that NKG2C^{brigth} NK cells in CMV seropositive blood donors co-expressed CD57 and mostly lacked NKGA and KIR3DL1 and degranulated stronger upon activation by plate bound antibodies (144). A subsequent longitudinal follow up of solid organ transplantation patients showed that NKG2C positive NK cells preferentially expand upon acute CMV infection and that these expanding cells acquire higher levels of NKG2C and co-expression of CD57 (144). Those so called "memory-" or "adaptive" NK cell subsets have also been observed upon CMV reactivation after SCT and are characterized by more potent effector function and a longer lifespan (145, 146). Direct evidence for a CMV-induced imprint of the NK cell compartment was obtained in *in vivo* studies showing that long-lived Ly49H positive NK cells, that were able to mount recall responses, preferentially expanded in mice upon MCMV infection (147). Moreover, *in vitro* studies showed that CMV-infected cells induce proliferation of NKG2C^{brigth} NK cells (148) although the molecular basis has not been fully resolved, interaction with HLA-E presenting CMV derived peptides is one of the main mechanisms suggested to drive the response (149, 150).

HLA-E is expressed by virtually every nucleated cell. Under normal conditions expression levels are low but like the classical HLA class I molecules, HLA-E cell surface expression can be enhanced under inflammatory conditions. Enhanced expression of HLA-E was observed in renal allograft biopsies in patients experiencing acute cellular rejection while this was not observed in patients without any signs of rejections (151). The higher levels of HLA-E were paralleled with increased numbers of CD8 and CD56 positive cells and a higher expression of NKG2C on these effector cells in renal tissue as well as in renal blood vessels. NKG2A was, in this study, almost completely absent on the effector cells suggesting a predominantly activating role for HLA-E in this setting and a role in deterioration of graft function and a higher risk of graft loss (151). Only one study directly evaluated the interaction between HLA-E and NK cells in the solid organ transplantation

setting showing that transgene expression of human HLA-E could protect pig endothelial cells in organs like the kidney and the heart against xenogeneic anti-pig cytotoxicity of human NK cells (152). HLA-E can bind peptides derived from several of the viruses known to give complications in the kidney transplantation setting as well as peptides derived from stress proteins. Moreover, HLA-E expression levels on the graft may be influenced by factors from the local microenvironment such as cytokines, DAMPs or PAMPs. Hence, it will be interesting to acquire a better understanding of the role of HLA-E in antiviral immunity after transplantation and rejection by more in depth analysis of HLA-E expression levels, -peptidome and the receptor repertoires of immune cells in the graft. To promote NK cell anti-viral immunity, blockade of NKG2A with HLA-E may be an interesting opportunity to explore, for example with clinically available monoclonal antibodies like monalizumab (153). Though, evidently, this should be tightly balanced to avoid graft rejection that may occur due to lower inhibitory effects of HLA-E.

HLA-F as Ligand for NK Cells Expressing KIR3DL2/LILRB1 and/or KIR3DS1

The HLA-F gene has a similar structure as the other HLA class I genes, except for the 3'untranslated region (154). Also exon 7 of the HLA-F gene remains untranslated leading to the production of shorter cytoplasmic tail than the other class I molecules (155). HLA-F is expressed intracellular in resting cells (156) and on the cell surface of certain cells including activated lymphocytes (157) and virus infected cells (158). On the cell surface, HLA-F has been found in two different forms; as a heterodimer with β2m and peptides, and as an open conformer without β2m and peptides (159, 160). The different conformations of the HLA-F molecule play an important role in determination of the type of NK cell receptor binding. It has been shown that open conformation binds to inhibitory KIR3DL2 and activating KIR3DS1 (159, 161, 162) while the heterodimer form binds to Ig like transcript (LILRB1 and LILRB2) (160). Although HLA-F has not been studied as intensively as HLA-E and G, increasing body of evidence reveal its clinical relevance in various pathologies including virus infection (163–165), pregnancy (166, 167), autoimmune diseases (168) and cancer (169, 170). However, until now, there are no investigations regarding the role of HLA-F in solid organ transplantation.

HLA-G as Immune Checkpoint for NK Cells Expressing KIR2DL4 and/or LILRB1

HLA-G is the most frequently studied non-classical HLA class I molecule in the kidney transplantation setting. In contrast to HLA-A,-B,-C and -E, HLA-G is expressed on a restricted set of cells and tissues and mainly in tissues characterized by a tolerogenic- or immune suppressive immune environment (171). Clear examples are the expression of HLA-G on trophoblast cells of the developing fetus during pregnancy or the enhanced expression of HLA-G on tumor or tumor-accessory cells contributing to immune evasion in cancer (172, 173). Also in the solid organ transplantation setting, HLA-G induced tolerance for the allograft has been described: In brief, HLA-G contributes to short- term tolerance by interacting with inhibitory receptors, like LILRB1/2 and KIR2DL4 on immune effector cells. Moreover, it contributes to long-term tolerance *via* the induction of tolerogenic-, IL-10 producing dendritic cells that promote regulatory T cells. In this review, we will focus on the effects of HLA-G on NK cells, the different mechanism of tolerance induction for other immune effector cells have been comprehensively summarized in (174, 175).

Like the other non-classical HLA class I molecules, the HLA-G gene displays only limited polymorphism as compared to HLA-A, -B, or -C. However, an important difference with the other non-classical HLA genes is the frequent occurrence of alternative splicing of the HLA-G gene leading to seven HLA-G isoforms that can be expressed either in a membrane bound- (HLA-G1-4) or in a soluble- (HLA-G5-7) form. Soluble isoforms are the result of alternative splicing of the transmembrane region encoded by exon 5 and can form HLA-G dimers that can signal more potently than monomeric variants (176, 177). The HLA-G1 and HLA-G5 isoform have three extracellular domains of the heavy chain and bind non-covalently to β2M. All the other isoforms express the α1 domain in combination with an α3 domain (HLA-G2 and HLA-G6), with an α2 domain (HLA-G4) or without any additional α domain (HLA-G3 and HLA-G7) (176, 178). Another unique feature is the HLA-G promotor that has a modified regulatory enhancer A (enhA) and lacks interferon-stimulated response elements (ISRE) making it unresponsive to NFκB and IFN-ϒ (179, 180). Consequently, HLA-G expression is not triggered by the typical stimulators of the other HLA class I genes which partly explains its' tissue restricted expression. HLA-G expression levels are also influenced by multiple SNPs in the promotor region, for example the -725G/T/C polymorphism in the promotor region results in higher HLA-G expression levels for -725G compared to -725C or -725T alleles (181). Given its important role in establishing and maintaining immune tolerance, HLA-G polymorphisms and levels of soluble HLA-G have been frequently determined as surrogate markers for several diseases and pathologies, and multiple studies demonstrated that high levels of membrane bound- or soluble HLA-G were associated with allograft acceptance and less occurrence of acute- or chronic graft rejection summarized in (175, 176).

In addition to the above mentioned mechanisms, HLA-G expression levels can be influenced by genetic variation in the 3'-untranslated region (3' UTR). Best characterized example, is the 14-base pair insertion or deletion fragment (14-bp INDEL) in exon 8 that, in 14-bp insertion allele variants, leads to the extra deletion of a 92-bp region at the start of exon 8 and enhanced mRNA stability (182). Another example is the C/G SNP on position +3142 that influences HLA-G targeting micro-RNA binding and, by doing so, mRNA stability (175). One of these micro-RNAs, miR365, is enhanced under hypoxic conditions (183) and it may be relevant to study its contribution to ischemia-reperfusion damage in the kidney transplantation setting. Moreover, the presence of the +3142CC genotype in kidney transplant recipients (n=178), as well as higher soluble HLA-G levels, have been associated with

higher susceptibility to CMV infection (184). A third example of the influence of genetic variation on HLA-G expression, is an A/G SNP on position +3187 that has been related to reduced HLA-G expression in +3187A alleles due to its proximity to the AU-rich motive (175). 3' UTR variation has been associated with multiple pathologies, including allograft rejection, where protective effects against rejection have been described for the 14-bp ins/ins and +3142 GG homozygous genotypes of the donor kidney (185). The linkage disequilibrium of the 14-bp ins/del and the +3187- and +3142 SNPs complicates analysis of the contribution of the individual regions to transplantation outcome. Hence, in a recent study, 3' UTR haplotypes were determined based upon fourteen 3' UTR SNPs (between +2960 and +3227) and the 14-bp ins/del (186). Subsequent deduction of the SNPs responsible for the observed effects, led to the identification of a +3003C SNP variant that, when present in donor as well as in recipient, was associated with BKPyV/PyVAN (Polyomavirus-associated nephropathy) occurrence and protection against antibody mediated rejection, while, the +3196G variant was associated with enhanced graft rejection (186).

Receptors for HLA-G are expressed on numerous immune cells including NK cells. LILRB1 (alias LIR1 or ILT2) and KIR2DL4 are the most well described receptors for NK cells. LILRB1 interacts with β2M-associated HLA-G molecules and exclusively acts as an inhibitory receptor for NK cells (187). KIR2DL4, one of the framework KIR genes, is predominantly expressed by decidual NK cells and both inhibitory- and activating-effects have been described upon interaction with HLA-G (188–191). Despite the numerous studies suggesting an important role for HLA-G to maintain allograft tolerance, the direct impact of HLA-G on NK cells in the kidney transplant setting or associations between HLA-G binding NK cell receptors is almost completely lacking. In a cohort of 81 healthy individuals *vs* 82 renal transplant recipients, a SNP in LILRB1 (rs1061680) has been shown to associate with increased carotid intimal media thickness (192) but additional comprehensive studies evaluating LILRB1 expression or genetic variation in kidney transplantation are lacking. By comparing 90 patients with a functional renal allograft and 40 patients rejecting their transplant, Ajith et al. confirmed the protective effect of high levels of the soluble HLA-G dimers (193). They also studied the underlying mechanism by genomic- and cellular analysis of patient derived T cells and in LILRB1 transgenic mouse models. This revealed that the HLA-G soluble dimers reduced the level of granzyme B in CD8 T cells in a LILRB1 dependent manner hence making them less cytotoxic. Although they did not address the role of NK cells in detail, a comparable mechanism may be relevant for the effect of soluble HLA-G on LILRB1 expressing NK cells as $CD8^+$ T cells, since NK cells use comparable mechanisms for target cell elimination.

NON-CLASSICAL HLA CLASS I DETERMINATION

Since typing of the classical class I molecules has been mandatory for transplantation purposes, many different DNA typing techniques and commercial kits are available. In contrast, typing of the non-classical HLA class I, HLA-E, -F and G genes, has been rather fragmentary.

For HLA-E, the typing method has long been limited to resolve the dimorphic amino acid at position 107 (R or G) by either PCR-SSP (PCR with Sequence-Specific Primers (194–197), PCR-SSO (PCR-Sequence Specific Oligo Probes (198), PCR-RFLP (PCR Restriction Fragment Length Polymorphism) alone (199) or in combination with ARMS (Amplification Refractory Mutation System) (200), PCR-SSCP (PCR-single strand conformation polymorphism) (201), Taqman assay (202) or sequence based typing of a limited part of the HLA-E gene either by Sanger sequencing (203–207) or recently also by NGS with Illumina (208). In this latter study HLA-E was typed for over 2.5 million potential stem cell donors worldwide and although only a limited 535 bp amplicon (including last part of exon 2, intron 2 and first part of exon 3) was sequenced, it has caused an explosion of new HLA-E alleles (209). Also full length sequencing of HLA-E was developed using both Sanger sequencing (210, 211) and NGS (212–216). These full length strategies have also revealed new alleles, including alleles with polymorphism present outside of the peptide binding groove. Although at present (IPD-IMGT/HLA database version 3.44.0) 271 different HLA-E alleles and 110 different HLA-E protein molecules have been recognized, the two major proteins HLA-E*01:01P (R107) and E*01:03P (G107) account for >99% of the population. In fact, in a huge study with > 2.5 million individuals typed for HLA-E, only in 0.05% another allele (01:05, 01:07 or new) was detected. Among the 110 different HLA-E protein molecules, there are only 2 that have an amino acid at position 107 that is different than the HLA-E*01:01/*01:03 main protein variants, namely HLA-E*01:48 (K107) and E*01:88 (S107). While the R107G SNP impacts HLA-E expression levels (126), the functional impact of the K107 and S107 change is not clear. Also the functional impact of amino acid differences at other positions, if any, is unclear. The 7 null alleles are all due to a single nucleotide difference, changing an amino acid coding codon to a stop codon (TAA, TGA or TAG) and all of them are located between positions 84 and 113, and therefore can easily be recognized if only part of the gene is sequenced.

HLA-F is the least polymorphic of the classical and non-classical HLA class I genes (A, B, C, E, F, G). In the present IPD-IMGT/HLA database (3.44.0) a total of 45 different alleles has been identified, but they only encode 6 different proteins. The amino acid differences are located at the start or the end of the protein, leaving the middle part (amino acids 51 – 250) identical for all hitherto known HLA-F alleles. There is also no null allele yet identified, implicating an important role for this conserved protein. Since there is only limited polymorphism identified, the methods to type for HLA-F have often been limited to the known polymorphism or to a fraction of the gene, enabling only identification of the known alleles. The method dealing with the known polymorphism that has been used for HLA-F typing is PCR-SSP (217), whereas in several studies part of the gene has been amplified and sequenced by Sanger sequencing (168, 204, 218). In more recent studies NGS of the whole gene has been

used for characterizing the HLA-F gene in several different populations (212, 219, 220) or as part of identification of all HLA genes in a multiplex set up (215).

Comparable to HLA-E and HLA-F also HLA-G exhibits few polymorphic sites along the sequence (221), although it might have attracted more attention because of its role in materno-fetal tolerance and the different splicing forms that have been identified, resulting in different soluble and membrane bound isoforms. At present there are 82 different HLA-G alleles in the IPD-IMGT/HLA database (3.44.0), encoding 22 different proteins, whereas 4 null alleles have been identified. Due to a rather conserved molecule more attention has been paid to analysis of the promotor region (5' upstream regulatory region, 5' URR) and the 3' UTR region that are thought to play a role in the expression levels of HLA-G and thus influence its immunotolerogenic (or immunomodulatory) properties (173, 222–226). Especially the 14 bp insertion/deletion that was identified in the 3' UTR region and correlated with mRNA stability and thus expression levels has been intensively studied by different methods, ranging from real time TaqMan PCR (227), amplification of part of the 3' UTR region followed by size discrimination analyzed by gel electrophoresis (228–230) to sequencing of this 3' UTR region (231–233). Not only 5' URR and 3' UTR are important, also the amino acid differences in the alpha2 domain have been found to influence the peptide binding repertoire resulting in functional differences between different HLA-G subtypes (234). Typing to identify the HLA-G alleles has mainly been performed in the framework of population and evolutionary studies and studies on reproduction, infection and disease associations, often limited to sequencing of exons 2-4 (230, 235, 236), sometimes combined with identification of the 14 bp insertion/deletion in the 3' UTR region (237–239). Recently, also NGS has been used to identify the HLA-G alleles present (212, 215, 240–242). A high linkage disequilibrium was found between the HLA-G allele type and the polymorphism in the 3' UTR region (241).

SHORT DISCUSSION/CONCLUSION

In the present review, we discussed the role of classical and nonclassical HLA class I molecules as immune checkpoints for NK cells and the relevance for two important determinants of kidney transplantation outcome: graft survival/rejection and viral infection. While several studies showed that expression levels- and soluble variants of non-classical HLA-E and -G are associated with rejection, data on the direct effects on NK cells and the contribution of NK cell receptors with specificity for non-classical HLA class I molecules in the kidney transplantation setting is rather limited. The contribution of KIR genes and iKIR-HLA class I matching status have been studied much more frequently. Multiple studies support the hypothesis that incompatibility between iKIRs in the recipient and HLA ligands in the graft may be detrimental for allograft survival. From the stem cell transplantation setting, it is becoming more and more clear that the impact of KIR-ligand matching *vs.* mismatching is greatly influenced by the exact transplantation protocol and beneficial effects of KIR-ligand mismatching were primarily seen in the severely T cell depleted setting (243). This may be explained by a reduced post-transplant pharmacological GvHD prophylaxis in this setting as such therapies have been shown to obscure the NK cells effects (244). Moreover, the exact model used to evaluate matching status could also influence the outcome as, in some studies, conclusions were drawn based on analysis of the presence *vs.* absence of HLA epitopes while in other studies this was complemented with data on the genotypic- or even phenotypic presence of the corresponding *KIR* genes. Since HLA and KIR are encoded on different chromosomes and KIR null alleles exist it would be relevant to address the relevance of KIR-ligand mismatching in larger cohorts using different models to determine matching status. In addition, functional studies could be used to further dissect the importance of KIR-ligand matching and this could also be related to the different immunosuppressive regimen. Most pronounced were the effects observed for the *KIR* gene repertoire and KIR haplotype and they were identified by various studies as important determinants of the immune response against the certain serotype of viruses. This illustrates that assessing genetic profiles of NK cell receptor with specificity for HLA class I may be useful to improve transplantation outcome. Given the dual role of NK cells in transplantation, it would be relevant to perform follow up studies to further evaluate the predictive value of those genetic profiles for combined endpoints, i.e. taking into account the occurrence of both graft rejection and infectious disease. The advancement in molecular typing methods for both HLA and NK cell receptors provides better discrimination of subtype of HLA alleles and specific types of KIR receptors and enables this type of analysis. Combined with functional- and spatial analysis of immune cell infiltration and -function and the identification of additional ligand-specificity for aKIR, this will facilitate the deeper understanding of the role of NK cell immune checkpoints in kidney transplantation, which may guide the exploitation of targeting NK cells for therapeutic benefits.

AUTHOR CONTRIBUTIONS

BD, TO, MG, CV, and LW wrote sections of the manuscript and reviewed it. All authors contributed to the article and approved the submitted version.

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Low Soluble Programmed Cell Death Protein 1 Levels After Allogeneic Stem Cell Transplantation Predict Moderate or Severe Chronic GvHD and Inferior Overall Survival

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Programmed cell death protein-1 (PD-1) is an inhibitory co-receptor required for regulating immune responsiveness and maintaining immune homeostasis. As PD-1 can be released as bioactive soluble molecule, we investigated the clinical significance of soluble PD-1 (sPD-1) after allogeneic hematopoietic stem cell transplantation (HSCT) regarding graft-versus-host disease (GvHD), relapse, and overall survival (OS) in a mono-centric cohort of 82 patients. Compared to pre-HSCT and to healthy controls, post-HSCT sPD-1 plasma levels were significantly increased during an observation time of three months. Univariate analysis revealed that low sPD-1 plasma levels at month one, two or three post HSCT were associated with acute GvHD grade III-IV, the onset of moderate/severe chronic GvHD (cGvHD) and inferior OS, DFS, and TRM, respectively. No relationship was detected to relapse rates. sPD-1 plasma levels were significantly increased in ATG-treated patients compared to ATG-untreated patients. Multivariate analysis revealed that a low sPD-1 plasma levels status at one or two month(s) after HSCT is an independent indicator for inferior OS, DFS, or TRM. A low sPD-1 plasma levels status at month three post HSCT is predictive for the onset of moderate/severe cGvHD. Thus, our study pinpoints the soluble inhibitory co-receptor PD-1 as a promising candidate molecule for the prediction of clinical HSCT outcome.

Keywords: allogeneic hematopoietic stem cell transplantation, graft-*versus*-host disease, PD-1, soluble PD-1, immune checkpoint, inhibitory co-receptor, GvHD biomarker

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a well-established cellular immunotherapy for a variety of malignant and non-malignant hematological diseases. The outcome of HSCT is determined by a balance of immune tolerance and immune alloreactivity. If the pendulum swings towards excessive immune alloreactivity, graft-versus-host disease (GvHD)

may result. If the pendulum swings towards exaggerated immune tolerance, the graft-versus-leukemia (GvL) effect, which is crucial for the prevention of relapse, is abrogated.

Immune checkpoint molecules function as physiological "brakes" of the immune system responsible for immune homeostasis (1). Inhibitory checkpoint ligands expressed on malignant cells enable tumors to escape anti-tumor immune responses. Specifically, the PD-1/PD-L1 signaling pathway can be hijacked as an immune escape mechanism in hematological malignancies (2). Programmed death-1 (PD-1, CD279) is expressed by activated CD4+ and CD8+ T cells, B cells, monocytes, dendritic and NK cells (3). PD-1 interacts with its cognate ligands PD-L1 (CD274) and PD-L2 (CD273). PD-L1 expression is upregulated by various tumor types including hematological malignancies.

With the advent of immune checkpoint inhibitors (ICI), a novel therapeutic modality of cancer immunotherapy has enriched the clinical armamentarium. Initially, ICI were deployed in solid cancers, but they are now increasingly used in hematological diseases and even after allogeneic HSCT. ICI after allogeneic HSCT are associated with the potentially severe or even lethal risk of inducing GvHD (4–6). A recent review summarizes that ICI is associated with GvHD not only if used after allogeneic HSCT, but also ICI prior to allogeneic HSCT is associated with acute GvHD (aGvHD) in 56% and with chronic GvHD (cGvHD) in 29% of patients (7).

There are only a few studies analyzing PD-1 expression after HSCT without checkpoint inhibition. Simonetta et al. (8) observed a significantly increased PD-1 expression on CD4+ and CD8+ T cells in 105 HSCT patients early after HSCT compared to healthy controls (HC). In the later course, the authors describe a progressive normalization of PD-1 expression on CD8+, but not on CD4+ T cells. The authors found no association of PD-1 expression on CD4+ and CD8+ T cells with donor/recipient matching, stem cell source, type of conditioning regimen and donor CMV sero-status. Noteworthy, T cell depletion (TCD) in general was significantly associated with elevated PD-1 expression on both CD4+ and CD8+ T cells. Since in this cohort various methods of in- and ex-vivo TCD were applied, the authors could discriminate that in-vivo TCD with ATG was associated with increased PD-1 expression on CD4+, but not on CD8+ T cells. In contrast, ex-vivo TCD with alemtuzumab or *in-vivo* TCD with post-cyclophosphamide were significantly associated with PD-1 upregulation on both CD4+ and CD8+ T cells. The authors conclude that these results might explain different effects of PD-1/PD-L1 blockade and also the associated different risk levels for GvHD depending on the time of administration. According to Jain et al. (9) PD-1 expression was elevated on T cells both in relapsed and non-relapsed patients after HSCT, indicating that membrane-bound PD-1 is not a dominant marker for leukemia-specific T cell exhaustion in the context of post-HSCT relapses.

Notably, PD-1 can be expressed as co-inhibitory receptor on cell surfaces, but it can also be released in soluble forms that can be detected in the plasma of respective patients. Soluble PD-1 is biologically active and can inhibit the interaction of membrane-bound PD-1 with PD-L1 and PD-L2 (10). Since none of the aforementioned studies, report on *soluble* PD-1 (sPD-1), we focused on sPD-1 in our monocentric study and investigated sPD-1 concentrations in plasma samples of 82 HSCT patients before and during the first three months after HSCT.

MATERIAL AND METHODS

Study Design

This prospective monocentric study was approved by the Ethical Board of the University Hospital of Essen (07-3503) and conducted in accordance to the Declaration of Helsinki. All patients gave their informed consent to participate in this study. Ethylenediaminetetraacetate (EDTA) plasma samples were serially collected from the patients before as well as 1, 2, and 3 month(s) post transplantation.

Patients' HSCT Disease Characteristics and GvHD Classification

Disease stage was classified according to the EBMT risk score for outcome after HSCT (11). Early disease stage included acute leukemia (AL) transplanted patients in first complete remission (CR), myelodysplastic syndrome (MDS) either untreated or in first CR, Non-Hodgkin's lymphoma (NHL) and multiple myeloma (MM) transplanted patients either untreated or in first CR; intermediate stage included AL in second CR, MDS in second CR or in partial remission (PR), NHL and MM in second CR, in PR or in stable disease. All other disease stages were considered as late stages.

Acute and chronic GvHD was categorized according to accepted standards (12–14). We grouped aGvHD grade 0-II as mild manifestations of aGvHD in contrast to aGvHD III-IV as severe manifestations of aGvHD. Acute and chronic GvHD were categorized according to the NIH 2005 criteria. Accordingly, we classified no or mild cGvHD as minor manifestations of cGvHD and compared these to moderate and severe cGvHD.

Quantification of Soluble PD-1

The determination of plasma levels of soluble PD-1 (sPD-1) was carried out as previously described (15) using a commercial ELISA kit (DuoSet ELISA Development System DY1086/CD279; R&D Systems, Wiesbaden-Nordenstadt, Germany) with minor modifications. Briefly, microtiter plates with high binding surface (Costar Corning, Bodenheim, Germany) were coated with anti-human PD-1 (842902 R&D Systems) at 4°C overnight at a final concentration of 1 µg/ml. Thereafter, free binding sites were blocked with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA, AppliChem GmbH, Darmstadt, Germany) and 0.05% Tween-20 (Carl Roth GmbH, Karlsruhe, Germany). Plasma samples were used undiluted and tested in duplicate. Recombinant PD-1 protein fused with the Fc-portion of human IgG (R&D Systems) was used as standard reagent and serially diluted in concentrations ranging from 0 to 10,000 pg/ml. Detection reagent of bound PD-1 (842903, R&D Systems) was used in a concentration of 50 ng/ml being diluted

in blocking buffer. Bound detection antibodies were recognized by streptavidin conjugated with horseradish peroxidase (R&D Systems) being diluted 1:200 in blocking buffer. The 3,3,5,5-tetramethybenzidine substrate reagent set (Becton Dickinson, Heidelberg, Germany) served as substrate. The substrate reaction was stopped by 2N H2SO4 and optical density was measured at 450 nm (Biotek Instruments, Winooski, VT, USA). Quantification of PD-1 plasma levels was performed by four-parameter curve fitting. Intra- and interassay coefficients of variation were 13.0% and 19%, respectively.

Flow Cytometry Analysis

Cell surface expression was analyzed by staining with fluorchromes-conjugated mononuclear antibodies against human CD3 (ECD, clone OKT3; Beckman Coulter, Krefeld, Germany) and PD-1 (AF488, clone EH12.2H7, BioLegend, Koblenz, Germany). Isotype-matched antibodies served as negative controls (BD Bioscience, Heidelberg, Germany). Samples were subjected to flow cytometry using a CytoFlexS cytometer (Beckman Coulter). Data acquisition of at least 200,000 events was performed with CytExpert Version 2.1 software (Beckman Coulter) and analyzed with Kaluza Analysis 2.1 software. Mean fluorescence intensity (MFI) index was defined by the ratio obtained from mean intensity of PD-1 staining on CD3 divided by the corresponding isotype matched control.

Statistics

Statistical analyses and presentation were performed by using SPSS 23.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism V8.4.3 software (GraphPad Software, San Diego, CA, USA). Data are presented either as median with range or as mean ± SEM (standard error of mean). After testing for Gaussian distribution, continuous variables were compared by T-test, non-parametric Mann-Whitney or two-way analysis of variance (Kruskal-Wallis test with uncorrected Dunn's test for multiple comparison), as appropriate. Nonparametric Spearman correlation was used to correlate the sPD-1 levels with PD-1 surface expression on CD3+ T cells. For categorical data, 2-sided Fisher's exact test was used. Clinical outcome endpoints of the study were overall survival (OS), disease-free survival (DFS), transplant-related mortality (TRM), acute graft-versus-host disease (aGvHD) grade III-IV, and moderate/severe chronic GvHD (cGvHD). OS was defined as time from HSCT to death from any cause. DFS was assessed as time from HSCT to treatment failure due to relapse, whereas TRM was assessed as time from HSCT to death without any sign of relapse. Using BIAS 11.08 software program (http://www.biasonline.de/) receiver operating characteristic (ROC) analysis was performed to define the optimal threshold value for sPD-1 regarding sensitivity and specificity to stratify the continouous parameter into a dichotomous variable for the prediction of aGvHD, cGvHD, OS, DFS, and TRM. Probabilities of OS and DFS were analyzed using the Kaplan-Meier method in combination with the log-rank test implemented in the R package survminer (version 0.4.0; https://CRAN.R-project.or/package=survminer). Stepwise multivariate Cox regression according to proportional hazards assumption was used to identify prognostic factors for OS and DFS, respectively. For moderate/severe cGvHD, aGvHD grade III-IV was defined as competing event. Relapse was the competing risk event for TRM. Univariate competing risk analysis by Aalen-Johanson-estimator and multivariate competing risk regression models were performed using BIAS 11.08 or STAT MP 16.0. Covariates were included into the multivariate analyses based on conceptual evaluation of literature or being associated with a p-value <0.05 to certain clinical parameters in univariate analysis. Statistical significance was defined as $p \leq 0.05$.

RESULTS

Patient Characteristics

Eighty-two patients, 42 female and 40 male, were enrolled in the study. Median age was 56 years (range 19-75 years). The majority (40 patients [pts.]) were diagnosed with Acute Myeloid Leukemia (AML). Other diagnoses included Myelodysplastic Syndrome (MDS, 8 pts.), Acute Lymphoblastic Leukemia (ALL, 8 pts.), Non-Hodgkin Lymphoma (NHL, 12 pts.), Myeloproliferative Neoplasms (MPN, 12 pts.) and other (2 pts.). These patients underwent HSCT between April 2017 and March 2019 at the Department of Hematology and Stem Cell Transplantation of the University Hospital Essen, Germany. Median CD34+ transplanted was 6.7 x 10^6/kg body weight (BW) of the recipient (range 3.0-19.5). The patients' and HSCT characteristics are detailed in **Table 1**. Fifty-six (68%) of the 82 patients received anti-thymocyte globulin (ATG) as *in vivo* T-cell depletion. Thirty-two patients received total body irradiation (TBI) as part of the conditioning regimen. Twenty-one patients received grafts from related donors; the remaining 61 patients received grafts from unrelated donors. In 73 cases the HSCT was HLA-identical, 9 patients were transplanted with an HLA-mismatched graft. Obviously, related vs. unrelated donors and GvHD prophylaxis were significantly different in the ATG-treated compared to the non-ATG-cohort. Median age, also, was significantly higher in the ATG-treated compared to the non-ATG-cohort. Besides, there were no significant difference in gender, diagnoses, CD34+ cells/kg BW, HLA-identical *vs.* mismatched, acute GvHD grade 0-II vs. III-IV, no/mild *vs.* moderate/severe chronic GvHD, relapse and OS when comparing the ATG- and the non-ATG-cohort (**Table 1**). At a median follow-up of 310 days (range: 22-791) after HSCT, 9 patients (11%) had suffered a relapse and 62 patients (76%) were alive.

The median onset of acute GvHD was 16 days (range: 10-65). In our cohort, all acute GvHD showed classical onset, there were no cases of late onset aGvHD. The distribution of maximal aGvHD was as follows: no aGvHD: 4; aGvHD I°: 53; aGvHD II°: 17; aGvHD III°: 7; aGvHD IV°: 1. Regarding chronic GvHD, 24 patients showed no symptoms of cGvHD; 2 pts. developed *de novo*-cGvHD without prior aGvHD; 23 pts. had quiescent cGvHD; 23 pts. suffered from progressive cGvHD out of aGvHD; data n.a.: 10. The median onset of chronic GvHD was 152 days (range: 100-690). The distribution of maximal cGvHD

TABLE 1 | Demographic and HSCT characteristics of patients.

Number of patients (N;%)	All patients	Non ATG-treated	ATG-treated*	p-value**
	82	26 (32%)	56 (68%)	
Median age [years(range)]	56 (19-75)	51 (20-69)	59 (19-75)	0.02
Gender (female/male)	42/40	13/13	29/27	n.s.
Diagnosis at allo SCT				n.s.
AML	40	12	28	
MDS	8	1`	7	
ALL	8	3	5	
NHL	12	4	8	
MPN	12	3	9	
Other	2	2	1	
CD34 x 10^6/kg BW[median(range)]	6.7 (3.0-19.5)	7.1 (3.1-13.3)	6.3 (3.0-15.0)	n.s.
Conditioning				n.s.
TBI(8-12 Gy) & Flu, cycloph or Etopos	32	11	21	
Fludarabine and Busulfan	28	7	21	
Fludarabine and Treosulfane	18	5	13	
Other	4	3	1	
Unrelated donor(URD) yes/no	61/21	7/19	54/2	<0.0001
HLA-identical yes/no	73/9	23/3	50/6	n.s.
Female to male HSCT yes/no	12/70	7/19	5/51	0.0451
Follow-up time [days(median,range)]	310 (22-791)			
GvHD prophylaxis				2
CSA & MTX	66	15	51	
CNI*** & MMF	14	11	3	
Other	2	0	2	
Acute GvHD****				
Onset acute GvHD(median, range)	16 (10-65)	16 (10-31)	16 (10-65)	n.s.
Acute GvHD grade 0-II(max. severity)	74	24	50	
Acute GvHD grade III-IV(max. severity)	8	2	6	n.s.
Chronic GvHD****				
Onset chronic GvHD(median, range)	152 (100-690)	191 (101-690)	142 (100-523)	n.s.
no/mild chronic GvHD(max. severity)	52	15	37	
Moderate/severe chronic GvHD(max. severity)	20	9	11	n.s.
Relapse (yes/no) N (%)	9/73 (11%/89%)	1/25 (1%/30%)	8/48 (10%/59%)	n.s.
Survival (yes/no) N (%)	62/20 (76%/24%)	21/5 (26%/6%)	41/15 (50%/18%)	n.s.

**All but one patient received ATG Neovii™ in a cumulative dosage of 30-60 mg/kg BW. One patient received Thymoglobulin Genzyme™ in a dosage of 6 mg/kg BW.*

*** Comparisons between patients treated with ATG and non-treated with ATG (Fisher's exact test or unpaired t-test); n.s., not significant.*

****CNI, CSA or Tacrolimus.*

*****GvHD not evaluated for all patients due to death/missing clinical data. Maximal severity for acute and chronic GvHD are indicated.*

was as follows: no or mild cGvHD: 52; moderate cGvHD: 9; severe cGvHD: 11; data n.a.: 10. Transplant-related mortality was categorized according to etiology either due to infection, organ toxicity and acute GvHD with the following results: TRM due to infection: 6; TRM due to organ toxicity: 5; TRM due to aGvHD: 6. The other three deaths were due to relapse of the hematological disease.

Increased Levels of sPD-1 Post HSCT

To study the effect of HSCT on the release of soluble PD-1 (sPD-1) molecules into the blood circulation, sPD-1 levels were compared among healthy controls (HC) and patients pre-HSCT and post-HSCT. sPD-1 levels of HC were not significantly different from the ones of pre-HSCT patients (**Figure 1**). Furthermore, pre-HSCT sPD-1 levels were not associated to patients' gender and disease (**Supplementary Figures 1A, B**). However, sPD–1 levels were significantly increased ($p<0.001$, $p<0.5$ and $p<0.01$) one, two and three months post-HSCT, compared to pre-HSCT levels; and overall, no substantial fluctuation of sPD-1 was observed within this observation period (**Figure 1**). Of note, an inverse correlation of post sPD-1 levels ($r=-0.4154$, $p=0.0095$) with the PD–1 intensity of cell surface expression on CD3+ T cells was observed in 13 patients post HSCT (**Figure 2**).

The Course of sPD-1 Levels Are Decreased in Patients With GvHD and Inferior OS, DSF, and TRM

To investigate the association of sPD-1 plasma levels with HSCT outcome, the course of sPD-1 levels was related to aGvHD, cGvHD, OS, DFS, TRM, and relapse (**Figure 3**). The sPD-1 levels (mean ± SEM, pg/ml) of 8 patients with severe aGvHD grade III-IV presented decreasing sPD-1 levels within the first three months post HSCT, whereas the sPD-1 levels of patients with aGHD grade 0-II (n=72) were higher during this observation time (**Figure 3A**). Significantly decreased sPD-1 levels ($p=0.0056$) were observed for 20 patients experiencing moderate or severe cGvHD compared to 52 patients with no or only mild cGvHD (**Figure 3B**). Among patients with moderate/severe cGvHD, the course of sPD-1 levels was not different between quiescient and progressive cGvHD (**Supplementary Figure 2**). Noteworthy, lower sPD-1 levels

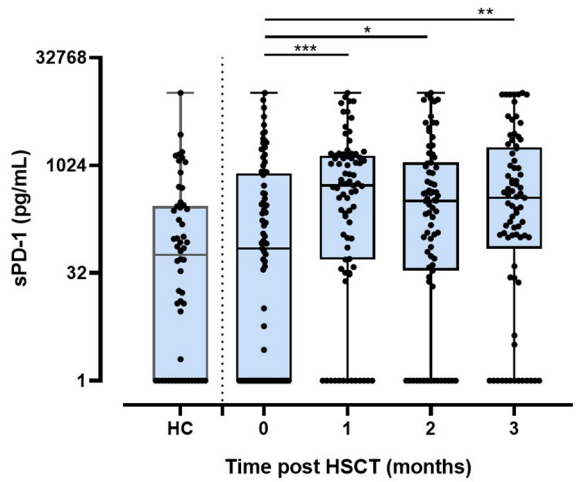

FIGURE 1 | Levels of sPD-1 in healthy controls and in patients before and post HSCT during the first three months. HC, healthy controls (n=54); 0: before HSCT; 1, 2, 3: one, two, three month(s) post HSCT; *p < 0.05, **p < 0.01; ***p < 0.001.

before and during the obervation time were significantly associated with inferior OS (p=0.0006, **Figure 3C**), DFS (p=0.0057, **Figure 3D**), and TRM (p=0.0099, **Figure 3E**) post HSCT. Even though patients who experienced disease recurrence displayed lower sPD-1 levels two and three months post HSCT compared with patients without a relapse, the course of these patients' groups appeared not to be significantly different (**Figure 3F**).

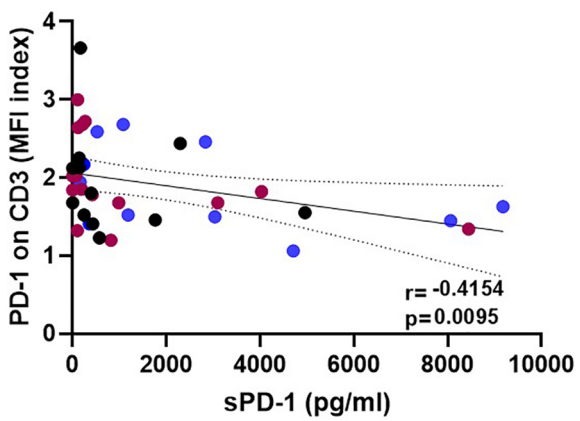

FIGURE 2 | Inverse correlation of post-HSCT sPD-1 levels with surface expression on CD3+ T cells. Plasma samples and corresponding peripheral blood lymphocytes were taken at various time points: blue, brown, and black dots correspond to 1, 2, and 3 month(s) post HSCT, respectively; solid line indicates regression line; dotted lines represent the corresponding 95% confidence bands; r: correlation coefficient value.

Low sPD-1 Levels at Month Two or Three Post HSCT Are Indicators for aGvHD Grade III-IV and the Onset of Moderate/Severe cGvHD

In order to identify a potential threshold level indicating an increased risk for severe aGvHD grade III-IV, receiver operating characteristic (ROC) analysis was performed (**Table 2**). A significant sPD-1 cut-off level of 461 pg/ml one month post HSCT was defined for the onset of moderate/severe cGvHD, whereas no relevant threshold values could be identified for aGvHD grade III-IV. Using sPD-1 cut-off level of 461 pg/ml, univariate competing risk analysis for moderate/severe cGvHD with aGvHD grade III-IV as competing event did not present statistically different cumulative incidence functions neither for cGvHD (p=0.059) nor for the competing risk aGvHD (p=0.116, **Supplementary Figures 3A, B**).

Two months post HSCT, a sPD-1 cut-off level of 133 pg/ml was significantly associated with both, aGvHD grade III-IV and the onset of moderate/severe cGvHD (**Table 2**). Here, univariate competing risk analysis did not present statistically different cumulative incidence functions for cGvHD (p=0.198, **Figure 4A**) but for the competing events aGvHD grade III-IV (p=0.002, **Figure 4B**), indicating that sPD-1<133 pg/ml is an predictor of aGvHD grade III-IV rather than for moderate/severe cGvHD at two months post HSCT. Three months post HSCT a sPD-1 cut-off level of 107 pg/ml (**Table 2**) was exclusively associated with the onset of moderate/severe cGvHD (p=0.011) by univariate competing risk analysis (**Figures 4C, D**). Taken together, low sPD-1 levels two or three months post HSCT are indicators for severe aGvHD or the onset of moderate/severe cGvHD.

Low sPD-1 Status at Month One or Two Post HSCT Is a Prognostic Co-Variate for Inferior OS, DFS, and TRM

Moreover, we asked whether sPD-1 plasma levels were associated with OS, DFS, and TRM. A cut-off level of sPD-1 < or > 567pg/ml obtained one month post HSCT (**Table 2**) was significantly associated with OS (p=0.045), whereas this threshold did not reach significance for DFS (p=0.100) and TRM (p=0.056). Kaplan-Meier probabilities of OS (p=0.004; log-rank HR: 5.11, 95% CI: 2.03 - 12.88) and DFS (p=0.014; log-rank HR: 3.29, 95% CI: 1.40 - 7.74) were significantly reduced for patients below this threshold value compared with patients above this level (**Figures 5A, B**). The univariate competing risk analysis for TRM with relapse as competing event revealed statistically different cumulative incidence functions for TRM (p=0.015, **Figure 6A**) but not for relapse (p=0.331, **Figure 6B**).

For the second month post HSCT, a sPD-1 threshold level of 415 pg/ml was identified for OS (p=0.008), DFS (p=0.030), and TRM (p=0.020) by ROC analysis (**Table 2**). Patients with sPD-1 values below 415 pg/ml showed inferior OS probability (p=0.001, log-rank HR: 13.13, 95% CI: 5.05 - 34.14) and inferior DFS (p=0.006; log-rank HR: 4.82, 95% CI: 2.10 – 11.02) with a median DFS of 498 days post HSCT as compared with patients above this cut-off (**Figures 5C, D**). Again, competing risk analysis showed

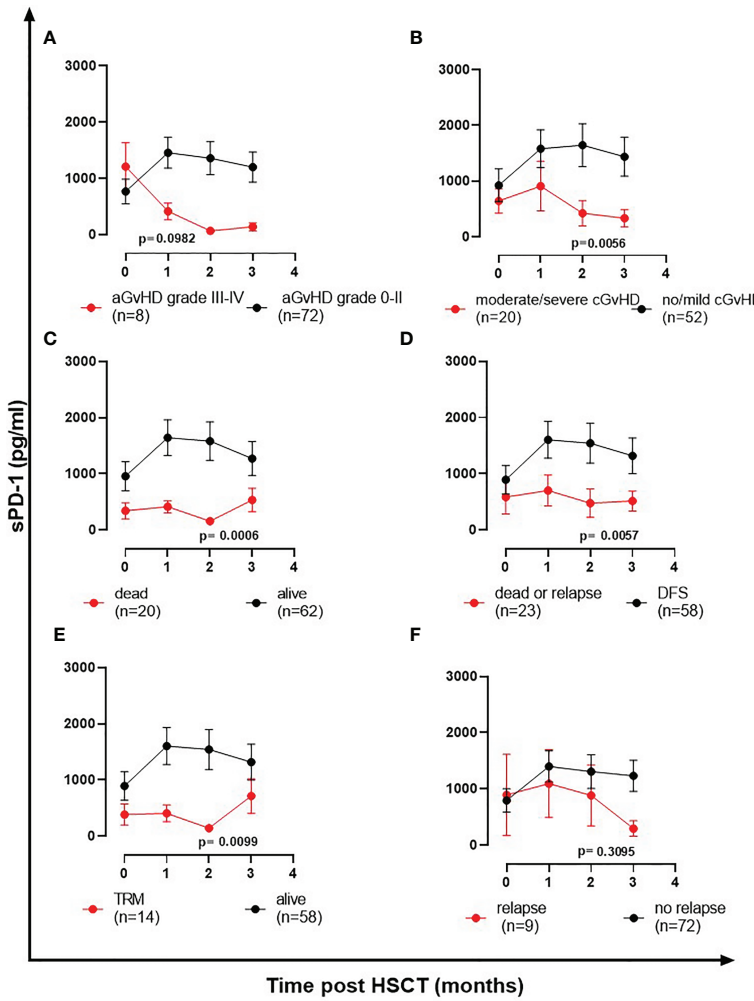

FIGURE 3 | The course of sPD-1 levels in relationship to clinical outcome post HSCT. Course of sPD-1 levels in patients with **(A)** aGvHD grade III-IV (red line) versus aGvHD grade 0-II (black line), **(B)** moderate/severe cGvHD (red line) versus no/mild cGvHD (black line), **(C)** patients passed away (red line) versus patients being alive (black line) at month three, **(D)** patients passed away or with relapse (red line) *versus* patients with disease-free survival (black line), **(E)** patients with transplant-related mortality (TRM) and not due to relapse (red line) versus patients being alive (black line), and **(F)** patients experiencing recurrence (red line) *versus* patients without relapse during the follow-up time. Data are presented as mean ± SEM before HSCT (0) and one, two, three month(s) post HSCT. Manifestation of aGvHD or cGvHD could not be evaluated for all patients due to death or missing clinical data.

different cumulative incidence functions for patients with sPD-1 < or > 415 pg/ml (p=0.011, **Figure 6C**) for TRM but not for the competing event relapse (p=0.189, **Figure 6D**).

sPD-1 Levels Are Significantly Increased in ATG-Treated Patients Post HSCT

To study the impact of conditioning regimens, patients were stratified into groups of ATG-treated (n=56) and ATG-untreated patients (n=25; one ATG-patient died within the first month post HSCT and hence was not included in the analysis). The sPD-1 levels were nearly 3-fold increased in ATG-treated patients in the first 3 months post HSCT compared to ATG-untreated patients (p=0.0015, **Figure 7A**). In contrast to ATG-treatment, total body irradiation during conditioning did not substantially impact (p=0.1844) the course of sPD-1 levels post HSCT (**Figure 7B**).

Multivariate Analyses Identifies Low sPD-1 Levels During the First Three Months Post HSCT as Independent Indicators for Moderate/Severe GvHD, Reduced OS and DFS and Increased TRM

All multivariate analyses encompassed disease status, ATG-treatment, age at time of HSCT, unrelated *vs.* related donor, female donor to male patient, GvHD prophylaxis (cyclosporine A [CSA] & methotrexate [MTX] versus calcineurin inhibitors (CNI) [either CSA or Tacrolimus] & mycophenolate mofetil [MMF]), and the sPD-1 status using the different cut-off levels for moderate/

TABLE 2 | sPD-1 cut-off levels defined by ROC analysis for aGvHD, cGvHD, OS, DFS, and TRM at month one, two or three post HSCT.

HSCT endpoint	Month post HSCT	Cut-off	AUC	Sensitivity	Specificity	p
aGvHD grade III-IV	1	1229	0.605	34.7	100.0	0.359
	2	133	0.744	64.7	100.0	0.048
	3	405	0.699	46.2	100.0	0.139
moderate/severe cGvHD	1	461	0.675	61.6	75.0	0.019
	2	133	0.690	90.0	68.9	0.015
	3	107	0.724	60.0	77.1	0.003
OS	1	567	0.655	83.3	57.4	0.045
	2	415	0.709	94.1	52.6	0.008
	3	489	0.517	78.5	41.3	0.841
DFS	1	567	0.621	76.2	56.2	0.100
	2	415	0.664	85.0	51.9	0.030
	3	1633	0.543	94.1	25.4	0.590
TRM	1	567	0.675	83.3	56.9	0.056
	2	415	0.723	90.1	51.8	0.020
	3	140	0.515	77.8	41.8	0.885

p-values were defined by Mann-Whitney test. AUC, Area under curve; aGvHD, acute Graft-versus-Host Disease; cGvHD, chronic Graft-versus-Host Disease; OS, overall survival; DFS, disease-free survival; TRM, transplantation-related mortality.

severe cGvHD and OS, DFS, TRM (**Table 2**), respectively, obtained during the observation period of three months post HSCT as co-variates. For OS and DFS, multivariate Cox regression revealed that the sPD-1 status with cut-off values of 567 pg/ml at one month was an independent indicator for OS (p=0.008, HR: 7.42, 95% CI: 1.69 - 32.48) and DFS (p=0.016, HR: 3.88, 95% CI: 1.29 - 11.63) post HSCT (**Figures 8A, B**). Similary, a cut-off value of sPD-1 of 415 pg/mL at two months post HSCT (**Figures 8D, E**) was an independent predictor for OS (p=0.016, HR: 11.99, 95% CI: 1.58 – 91.00) and for DFS (p=0.026, HR: 4.11, 95% CI: 0.96 – 5.91). Concerning TRM with relapse as competing event a sPD-1 <567 pg/ml one month post HSCT was found to be a significant independent indicator (p=0.043, SHR: 2.35, 95% CI: -0.12 – 4.59; **Figure 8C**), whereas sPD-1 <415pg/ml two months post HSCT did not reach significance (p=0.061, SHR: 2.08, 95% CI: -0.09 – 4.25; data not shown). Competing risk regression analysis revealed that a cut-off level of sPD-1 <107 pg/ml at month three post HSCT was an independent predictor for the onset of moderate/severe cGvHD

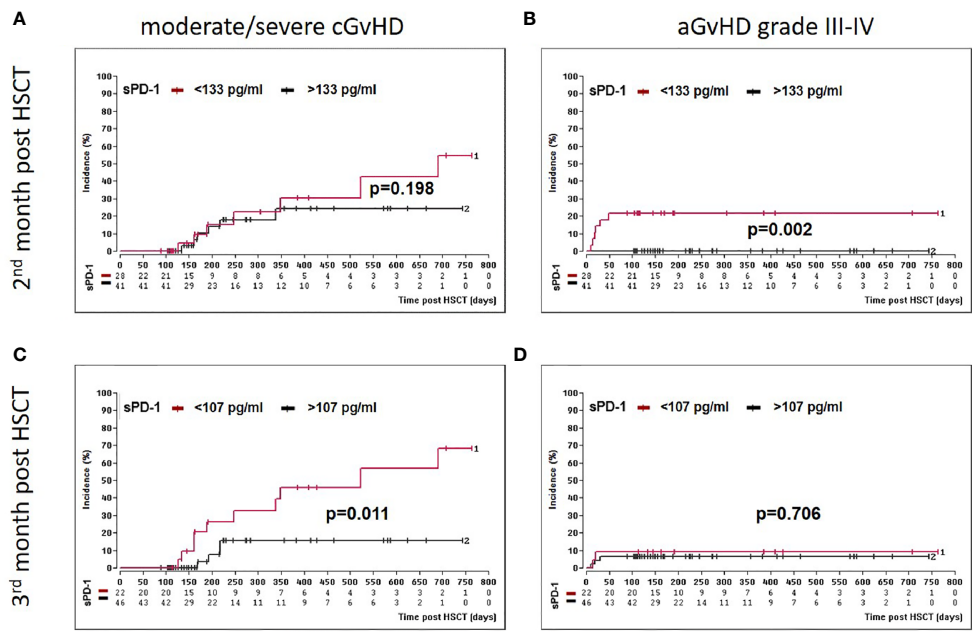

FIGURE 4 | Association of low sPD-1 level with aGvHD grade III-IV and moderate/severe cGvHD. Patients were divided into two groups according to cut-off levels [(< or >133 pg/ml, **(A, B)**] or [(> or <107 pg/ml, **(C, D)**] obtained two **(A, B)** and three months **(C, D)** post HSCT, respectively. Estimated cumulative incidence curves of patients with moderate/severe cGvHD **(A, C)** and aGvHD grade III-IV **(B, D)** as competing event are shown for patients with sPD-1 <133 pg/mL **(A, B)** or <107 pg/ml **(C, D)** in brown and compared to patients with >133 pg/mL(A, B) and >107 pg/ml **(C, D)** in black. The sPD-1 levels were available only for 69 patients **(A, B)** at month two or 68 patients **(B, C)** at month three post HSCT due to death or loss of follow up.

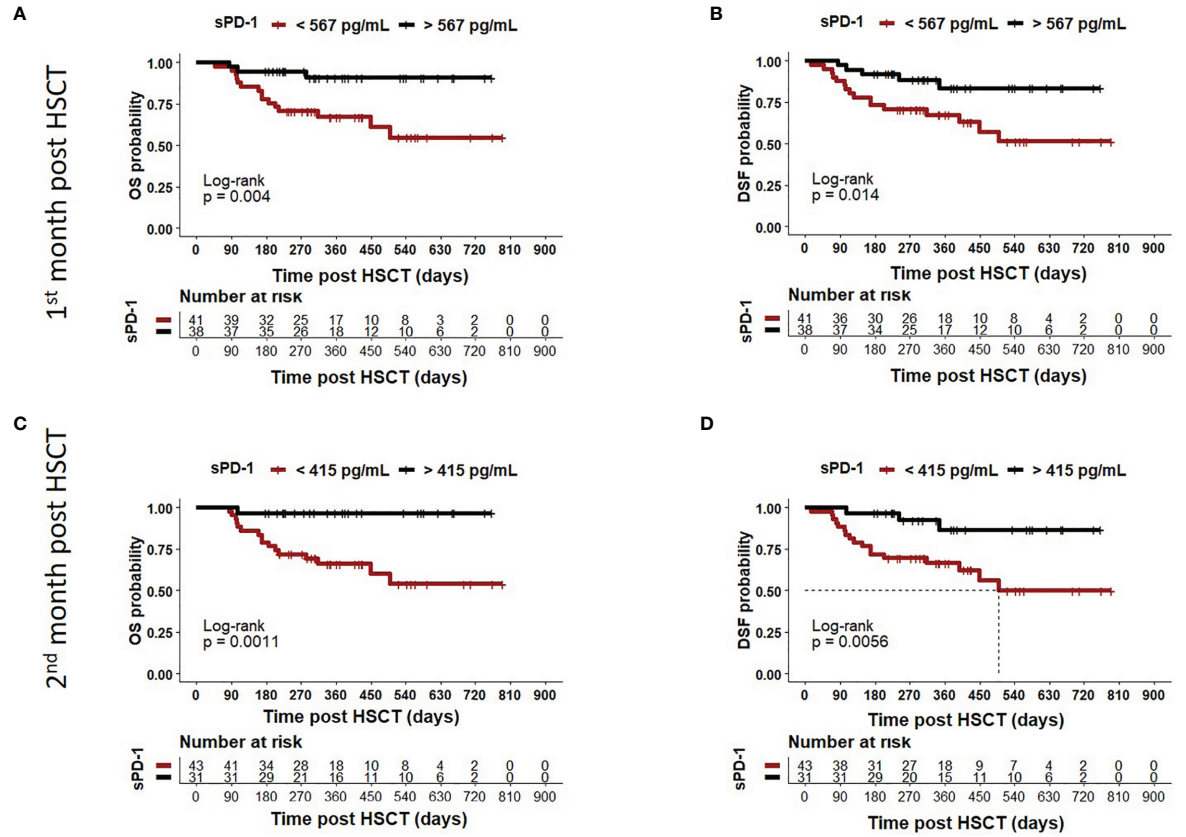

FIGURE 5 | Association of low sPD-1 levels with reduced OS and DFS. Patients were divided in two groups according to cut-off levels obtained one **(A, B)** month (< or >567 pg/ml) and two **(C, D)** months (< or >415 pg/ml) post HSCT. The corresponding Kaplan-Meier curve of overall survival (OS) or disease-free survival (DFS) probability combined with Log-rank test with respect to sPD-1 cut-offs are shown. Patients with sPD-1 below the thresholds in brown showed reduced OS (p=0.004 or p=0.011) and DFS (p=0.0014 or p=0.0056) compared with patients with sPD-1 levels above these values. Dotted line indicates median DFS post HSCT in **(D)**. Due to death or loss of follow up, data were not available for all 82 patients.

(p=0.031, Subhazard ratio (SHR): 3.45, 95% CI: 1.12 – 10.64) with aGvHD grade III-IV as competing event (**Figure 8F**).

DISCUSSION

The two most important complications after HSCT are relapse of the underlying hematological disease and graft-versus-host disease. Both complications are mainly determined by immunological processes: Loss of the desired anti-malignancy potency of donor-derived immune cells is one of the major reasons for relapse. In contrast, severe GvHD is induced by an excessive alloreactivity of donor-derived immune cells against patient's healthy tissue. Thus, a sound homeostasis of immune activity and immune tolerance is essential for the success of HSCT.

In recent years, immune checkpoints as physiological mediators of immune regulation have gained increasing attention. We here present data of *soluble* PD-1 levels after HSCT and its association with the major relevant outcomes after HSCT. In summary, we observed significantly increased levels of sPD-1 in the plasma of patients after HSCT compared to healthy controls and to the pre-HSCT levels. In the plasma of patients with severe acute GvHD grade III-IV, of patients with moderate/severe chronic GvHD, of patients with inferior OS and DFS, as well as with increased TRM, sPD-1 levels were substantially decreased. A sPD-1 status <133 pg/ml at month two after HSCT was associated with aGvHD grade III-IV, and at month three a sPD-1 status <107 pg/ml was found to be exclusively associated with moderate/severe cGvHD in uni- and multivariate analysis. Levels of sPD-1 below the cut-off values defined by ROC analysis for month one and two post HSCT indicated a significantly reduced OS and DSF probability and an increased TRM. Regarding PD-1 prediction of cGvHD associated mortality independently of aGvHD, our data do not support the assumption that the association of cGvHD associated mortality can be viewed independently from aGvHD, since in most cases severe manifestations of cGvHD develop out of severe cases of aGvHD. These are rather continuous manifestations than independent states. This is also supported by the fact that there is no difference in sPD-1 levels

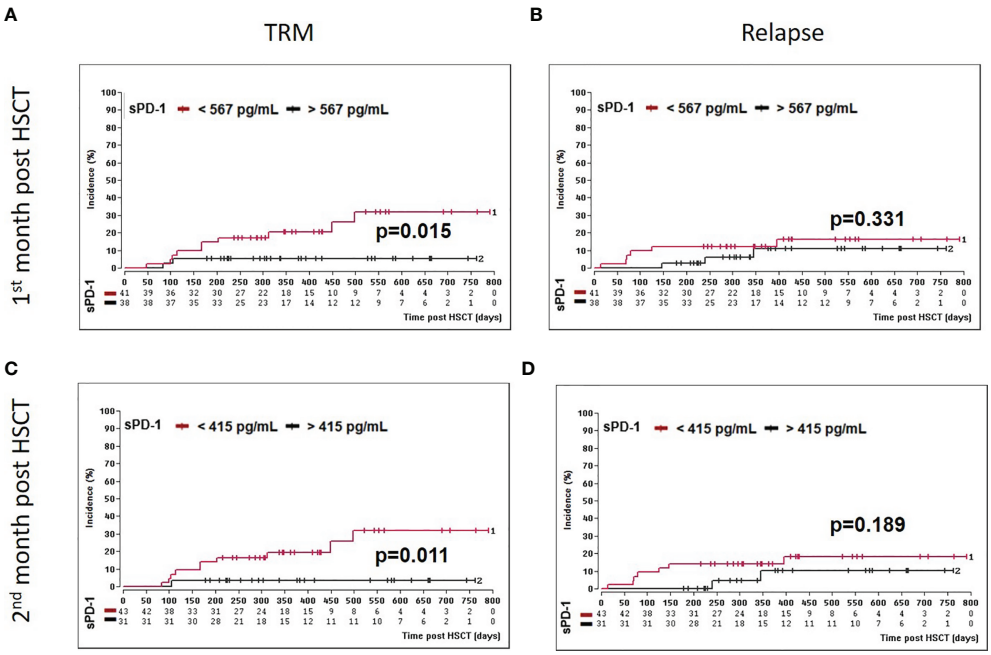

FIGURE 6 | Association of low sPD-1 levels with reduced TRM. Patients were divided in two groups according to cut-off levels obtained one **(A, B)** month (< or >567 pg/ml) and two **(C, D)** months (< or >415 pg/ml) post HSCT. Estimated cumulative incidence curves of patients with TRM **(A, C)** and relapse **(B, C)** as competing event are shown for patients with sPD-L1 < 567 pg/mL **(A, B)** or <415 pg/ml **(C, D)** in brown and compared to patients with sPD-1 >567 pg/mL **(A, B)** or >415 pg/ml **(C, D)** in black.

between moderate/severe cGvHD cases of quiescent *vs.* progressive type (**Supplementary Figure 2**). In multivariate analysis, low sPD-1 levels in plasma samples at month one or two after HSCT were confirmed as an independent predictive marker for reduced OS and DFS or increased TRM. Finally, in our cohort sPD-1 plasma levels were nearly 3-fold increased in ATG-treated patients in the first three months post HSCT compared to ATG-untreated patients. Notably, no difference was detected among plasma samples of patients treated with or without total body irradiation.

The published data on soluble PD-1 and its interaction with PD-1 expressed on cell surfaces is scarce and has not been

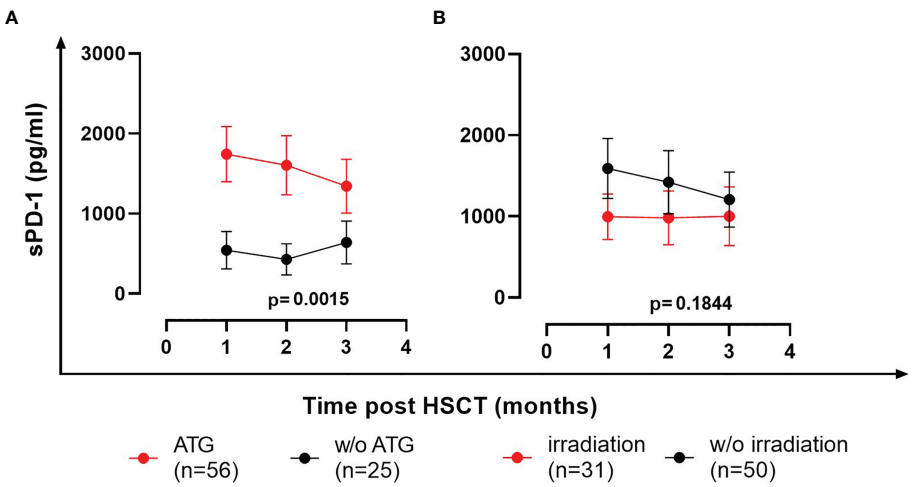

FIGURE 7 | Effects of ATG **(A)** and total body irradiation **(B)** on the course of sPD-1 levels post HSCT. Data are presented as mean ± SEM. sPD-1 levels of treated and untreated (w/o) patients are shown in red and black, respectively. One ATG-patient died within the first month post HSCT and hence was not included in the analysis.

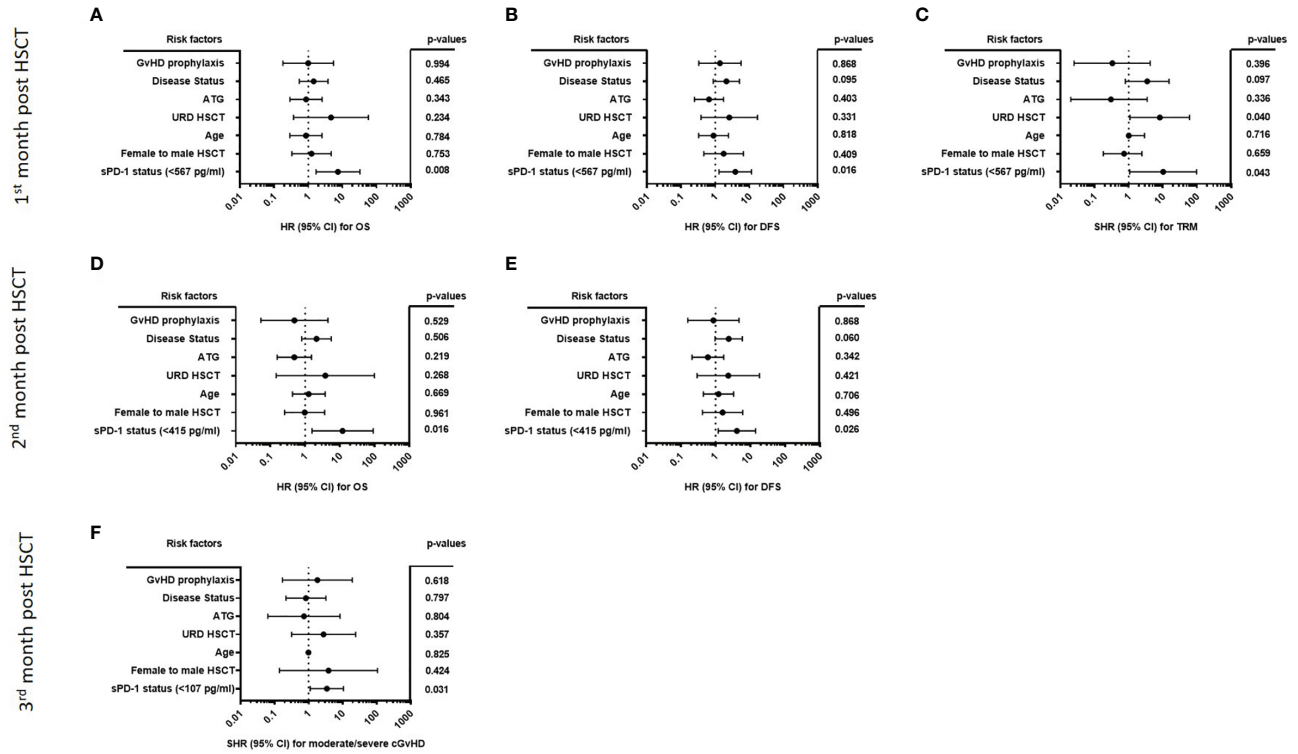

FIGURE 8 | Forest plot of risk factors for moderate/severe cGvHD, OS, DFS, and TRM post HSCT. The forest plots visualize the multivariate analyses of the following parameters for OS **(A, D)**, DFS **(B, E)** TRM with relapse as competing event **(C)** and moderate/severe cGvHD with aGvHD grade III-IV as competing event **(F)**: disease status (early/intermediate versus late), ATG-treatment (yes *versus* no), age at time of HSCT, URD-HSCT (yes *versus* no), female donor to male patient HSCT (yes *versus* no), GvHD prophylaxis (CSA & MTX *versus* CNI & MMF), and the sPD-1 status using the different cut-off levels. For OS, DFS, and TRM a cut-off of sPD-1 < 567 pg/ml of one month **(A–C)**, and a cut-off of sPD-1< 415 pg/ml two months post HSCT **(D, E)** were used, while for moderate/severe cGvHD a cut-off of sPD-1 < 107 pg/ml three months post HSCT was used. Due to death or loss of follow up, data were available for 74 **(A–C)**, 69 **(D, E)**, and 67 **(F)** out of 82 patients; 95% CI, 95% confidence interval; HR, hazard ratio; SHR, Subhazard ratio.

investigated in the setting of post-HSCT. sPD-1 is mainly thought to be produced by proteolytic cleavage of membrane-bound PD-1. Specifically, four different splice variants have been described while only one (PD-1Deltaex3) is likely encoding the soluble form of PD-1 (16). Increased levels of soluble PD-1 have been associated with advanced disease stage and – although not consistently – with worse prognosis in several malignancies (reviewed in (10)). Indeed, in this study a high variability of sPD-1 levels was observed for healthy controls and for pre/post HSCT patients. Furthermore, a wide range of cut-off values was defined for aGvHd/cGvHD and OS/DFS/TRM. Thus, further investigation are needed to clarify mechanisms of sPD-1 release into circulation and its functional consequences in view of aGvHD/cGvHD and OS/DFS/TRM post HSCT.

This study analyzes the course of sPD-1 levels prior to allogeneic HSCT and in the first three months after HSCT. Despite being a prospective study, it has several limitations. Firstly, the results of this mono-centric patient cohort of 82 patients need to be validated in larger and also multi-center studies. As we were able to show an inverse correlation between PD-1 expression on T cells and sPD-1 levels in peripheral blood only for a limited number of patients post HSCT, the relationship of membrane-bound and soluble PD1 has to be further elucidated especially in view of the functional and clinical relevance for HSCT outcome. Secondly, the influence of medication, notably immunosuppressive agents, and of infections on the course of membrane-bound and soluble immune checkpoints and their interactions have to be studied. Finally, the functional role of sPD-1 for the clinical HSCT outcome remains to be further elucidated. Interestingly, it has been reported that sPD-1 molecules are able to inhibit dendritic cell (DC) mediated CD4+ T cell activation and Th1 and Th2 cytokine production *via* the interaction of sPD-1 with PD-L1/2 expressed on DC, which results in a reduced expression of maturation marker and a suppressive DC phenotype (17).

In conclusion, the results presented here provide substantial evidence that soluble PD-1 could be a promising novel biomarker predicting severe GvHD and inferior survival after HSCT and opens up the discussion about the functional role of sPD-1 molecules in HSCT. In addition, we observed the influence of conditioning on the plasma levels of sPD-1, as sPD-1 plasma levels were nearly 3-fold increased in ATG-treated patients. Hence, further studies could help to evaluate the influence of immunosuppression and infections on sPD-1

levels and refine conditioning regimens to further improve HSCT outcome.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Board of the University Hospital of Essen, Germany (07-3503). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VR and LK conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. UB, FH, BG, PH, DB, and HR confirmed the analyses and assisted in correcting the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.694843/full#supplementary-material

Supplementary Figure 1 | No association of pre-sPD-1 levels with gender and disease type. sPD-1 levels are shown by Box-Whisker plot with minimum and maximum.

Supplementary Figure 2 | Course of sPD-1 levels in patients with progressive and quiescent type of moderate /severe cGvHD. Due to the low number of patients (n=1), data for sPD-1 levels with de novo type of moderate/severe cGvHD were not shown.

Supplementary Figure 3 | Association of low sPD-1 level with moderate/severe cGvHD and aGvHD grade III-IV at month one post HSCT. Patients were divided into two groups according to cut-off levels (< or >461 pg/ml) obtained one month post HSCT. Estimated cumulative incidence curves of patients with moderate/severe cGvHD **(A)** and aGvHD grade III-IV **(B)** as competing event are shown for patients with sPD-1 <461 pg/mL in brown and compared to patients with >461 pg/mL in black. The sPD-1 levels were available only for 73 out of 82 patients due to death or loss of follow up.

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ORIGINAL RESEARCH
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The Association Between Single-Nucleotide Polymorphisms of Co-Stimulatory Genes Within Non-HLA Region and the Prognosis of Leukemia Patients With Hematopoietic Stem Cell Transplantation

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To avoid graft rejection, the hematopoietic stem cells with matched classical human leukocyte antigen (HLA) alleles are the primary choice for clinical allogeneic transplantation. However, even if the fully HLA-matched hematopoietic stem cells are used for transplantation, some patients still have poor prognosis after hematopoietic stem cell transplantation (HSCT), suggesting that the HLA system was not the only determinant of the outcomes of HSCT. In this study, we investigated whether the single-nucleotide polymorphisms (SNPs) of the co-stimulatory genes within non-HLA regions were related to the outcomes of HSCT. The genomic DNAs of 163 patients who had acute leukemia and received HSCT and their respective donors were collected for analysis. Thirty-four SNPs located in the four co-stimulatory genes including cytotoxic T-lymphocyte associated protein 4 (CTLA4), CD28, tumor necrosis factor ligand superfamily 4 (TNFSF4), and programmed cell death protein 1 (PDCD1) were selected to explore their relationship with the adverse outcomes after transplantation, including mortality, cytomegalovirus infection, graft-versus-host disease, and relapse. Our results revealed that nine SNPs in the CTLA4 gene, five SNPs in the PDCD1 gene, two SNPs in the TNFSF4 gene, and four SNPs in the CD28 gene were significantly associated with the occurrence of adverse outcomes post-HSCT. These SNPs may play important roles in immune response to allografts post-HSCT and can be the targets for developing strategy to identify appropriate donors.

Keywords: hematopoietic stem cell transplantation, single-nucleotide polymorphism, non-HLA, CTLA4, CD28, TNFSF4, PDCD1 (PD-1)

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INTRODUCTION

Leukemia is a type of cancer with abnormal blood cells. It can be classified into myeloid and lymphoid lineage depending on the type of aberrantly multiplying cells. It can also be distinguished as acute or chronic according to the rate of disease progression. Acute leukemia has the characteristics of poor survival rate. Nowadays, the main treatment of acute leukemia is hematopoietic stem cell transplantation (HSCT), enabling reconstruction of the immune and hematopoietic systems by transplanting autologous or allogeneic hematopoietic stem cells into patients (1). Human leukocyte antigen (HLA) genes are located on the short arm of human chromosome 6, playing vital roles in immune response to allografts (2, 3). Hence, it is mandatory to confirm that the HLA alleles are matched between recipients and donors before transplantation (3–5), especially the classical HLA genes, such as HLA-A, -B, -C, and -D (-DR, -DQ, -DP). Because these genes are closely linked to each other, the HLA genes are inherited in the form of haplotype (6).

According to the relationship between the donor and recipient, HSCT can be divided into related and unrelated transplantation. In general, the survival rate of the former was higher than that of the latter. The incidence of adverse outcomes, such as acute graft-versus-host disease (GVHD), for related transplantation was generally lower than that of the unrelated transplantation (7, 8). GVHD is one of the complications of allogeneic HSCT caused by the donor's T cells attacking organs and tissues of recipients (9). Cytomegalovirus (CMV) infection is also a complication of allogeneic HSCT, resulting from the destroyed immune system in patients receiving high-dose treatment regimens (10). Patients with CMV infection and GVHD usually had a higher risk for recurrence and death (11). The allografts obtained from HLA-matched siblings was better than that obtained from the HLA-haploidentical parents, siblings, or unrelated sources (7, 12, 13). Because the probability of having HLA-matched siblings is 25%, there is only about a one-third chance to obtain an HLA-matched related donor. Most patients can only rely on public donation. Nevertheless, the source of donation is limited and patients usually are not able to acquire a suitable donor in time. Because graft failure still occurs even when an HLA-matched sibling was chosen as the donor, additional factors beyond HLA are likely to be involved in the regulator of allograft rejection (14, 15).

The association between HSCT and non-HLA genes such as tumor necrosis factor ligand superfamily 4 (TNFSF4), cytotoxic T-lymphocyte associated protein 4 (CTLA4 or CD152), programmed cell death protein 1 (PDCD1, PD-1 or CD279), and CD28 has been shown in previous studies (16–19). These genes belong to the co-stimulatory system. The imbalance of co-stimulatory molecules is one of the immune escape mechanisms in hematological cancers. It may promote the development of various autoimmune diseases and cancers (20). Several studies indicate that CD28, CTLA4, TNFSF4, and PDCD1 play important roles in the immune system and transplantations (21–23). In addition, genetic variants such as single-nucleotide polymorphisms (SNPs) in HLA and non-HLA regions have been linked to the success or failure of HSCT among different ethnic populations (24, 25). In this study, we explored the association between donor SNPs of co-stimulatory genes (CTLA4, CD28, TNFSF4, and PDCD1) and the mortality, CMV infection, GVHD, and relapse of their corresponding recipients in the Taiwanese population. This study provides new insights into understanding the roles of co-stimulatory genes in prognosis after transplantation and may lead to developing strategy to identify appropriate donors for HSCT.

METHODS AND MATERIALS

Patients and HLA Typing

This study was reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital, and its approval IDs were 201304949B0, 201700769B0, 201701849B0, 201801985B0, and 201901246B0. All donors and recipients except the donors of unrelated HSCT signed informed consents. All methods of the study were performed according to the ethical requirements and regulations. For unrelated donors, informed consents were exempted because the HLA-matched donors were selected by the Stem Cells Center in Taiwan and the identity of the donors were made anonymous and disconnected to the physicians and research team. A total of 163 patients receiving HSCT was enrolled in this study, in which 99 patients were diagnosed as acute myeloid leukemia (AML), and 64 patients were acute lymphoblastic leukemia (ALL). The clinical characteristics of the 163 patients are shown in **Table 1**. All donor–recipient pairs had fully matched HLA as revealed by high-resolution HLA typing using the SeCore kit (Thermo Fisher, Waltham, MA). The MicroSSP Allele Specific Typing Tray (Thermo Fisher, Waltham, MA) was used to resolve ambiguous alleles of the SeCore typing with sequence-specific primers.

Definition of Outcomes

Mortality was referred to the state of patients who died in the duration of study. The presence of CMV antigen or DNA in the peripheral blood of recipients after transplantation was defined as a CMV-infected case. CMV antigen in the leukocytes was determined by CMV Antigenemia Assay (MONOFLUO™, Bio-Rad). The test was considered positive when more than two polymorphonuclear leukocytes (PMN) were positive for CMV antigen in a total of 50,000 PMN. CMV DNA Quantitative Amplification test is a real-time quantitative PCR assay (COBAS® AmpliPrep/COBAS® TaqMan® CMV Test, Roche). The nucleic acid test was considered positive when the Ct < 37. These two assays can assist clinicians in monitoring the status of CMV infection.

According to the International Bone Marrow Transplant Registry, GVHD was considered as acute GVHD (aGVHD) when it occurred within 100 days after transplantation. It can be divided into four grades according to the clinical characteristics of organs as defined below. Grade I: maculopapular rash over <25% of body area with no liver or gastrointestinal involvement; Grade II: maculopapular rash over 25% to 50% of body area, diarrhea 500 to 1500 ml/day, and bilirubin 2 to 6 mg/dl; Grade III:

TABLE 1 | Clinical characteristics of patients who enrolled in the study.

Clinical features	No. of patients (%)	No. of AML patients (%)	No. of ALL patients (%)
No. of patients	163	99	64
Median age (years, range)	28 (0.7–66)	33 (1–66)	21 (0.7–54)
Gender (male/female)	75 (46)/88 (54)	42 (42)/57 (58)	33 (52)/31(48)
Mortality	83 (51)	51 (52)	32 (50)
CMV infection	88 (54)	62 (63)	26 (41)
Relapse	71 (44)	47 (47)	24 (38)
Acute GVHD I–II	48 (29)	25 (25)	23 (36)
Acute GVHD III–IV	11 (7)	7 (7)	4 (6)
Chronic GVHD	90 (55)	58 (59)	32 (50)
No GVHD	14 (9)	9 (9)	5 (8)

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CMV, cytomegalovirus; GVHD, graft-versus-host disease.

maculopapular rash over >50% of body area, and severe diarrhea; Grade IV: skin blisters, bilirubin >15 mg/dl, severe diarrhea with pain, and life-threatening. Grades I–II were defined as mild GVHD, and Grades III–IV were defined as severe GVHD. Chronic GVHD (cGVHD) usually occurs more than 100 days after transplantation or occurs continually for more than 100 days without remission (26). Patients without any symptoms of aGVHD or cGVHD during the study period were defined as no GVHD.

Relapse was defined as recurrence of malignancy based on one or more of the following: bone marrow morphology, minimal residual disease by either flow cytometry, cytogenetics, imaging results, or short tandem repeat (STR) analysis. High-throughput amplicon sequencing (AmpFISTR Identifiler Amplification Kit, Thermo Fisher, Waltham, MA) was performed to analyze STR and to evaluate HSCT engraftments for identification of mixed chimerism (27–29) according to the manufacturer's instruction. The presence of >5% recipient STR alleles in the chimeric test was considered as a surrogate marker of disease relapse.

Selection of SNPs

Based on our initial screening of SNPs that were present in the CTLA4 gene of the Taiwanese population, and the studies demonstrating the importance of promoter and exon 1 in gene expression and the SNPs with clinical association (30), a total of eight DNA fragments of the four co-stimulatory genes (CTLA4, TNFSF4, CD28, and PDCD1) were selected for analyses of donor SNPs (**Table 2**). A total of 17 SNPs in CTLA4, 3 SNPs in TNFSF4, 9 SNPs in CD28, and 5 SNPs in PDCD1 were subject to association study with the risk for relapse, mortality, GVHD, and CMV infection. All SNP variants were deposited to the NCBI database dbSNP and the accession numbers are provided in the **Supplementary Table S1**. Data can be accessed with the following link: https://www.ncbi.nlm.nih.gov/SNP/snp_viewTable.cgi?handle=WANGWT.

PCR and Sequencing

Peripheral blood (3 ml) was collected from the corresponding donors of the recipients, and the genomic DNA was extracted by

TABLE 2 | The 34 SNPs for association study with post-HSCT adverse outcomes.

Gene	Genomic region	SNP under analysis			
CTLA4	Promoter	rs11571315 rs62182595 rs5742909	rs733618 rs573554201	rs4553808 rs16840252	rs11571316 rs945677329
	Exon 1	rs231775			
	Exon 4	rs56102377	rs56217811	rs55696217	
	3'-UTR	rs231721	rs778932058	rs3087243	rs11571319
TNFSF4	Promoter	rs1234314	rs45454293	rs181758110	
CD28	Promoter	rs1879877 rs28718975 rs200353921	rs3181096 rs28688913	rs3181097 rs28541784	rs3181098 rs20189072
PDCD1	Promoter	rs5839828	rs36084323		
	Intron 4	rs41386349	rs6705653		
	Exon 5	rs2227982			

using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, California, USA). A total of eight primer pairs (**Table 3**) were used to amplify genomic DNA fragments covering the 34 SNPs as abovementioned (**Table 2**). The PCR reaction volume was 25 µl, including 1 µl of forward and reverse primer (10 µM), 12.5 µl of 2X HotStart PCR Mix (BIOMAN, Taipei, Taiwan) containing HotStart Taq DNA polymerase, reaction buffer, and dNTP, 1 µl of sample DNA and 9.5 µl of ddH_2O. The PCR program was 1 cycle at 94°C for 4 min, 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and 1 cycle of 72°C for 10 min. Subsequently, 5 µl of PCR products was fractionated on a 1.5% or 2% agarose gel to visualize the PCR products. The size of PCR products ranged from 1,039 bp to 2,234 bp (**Table 3**). The Big Dye Terminator Cycle Sequencing kit (Thermo Fisher, Waltham, Massachusetts, USA) and the ABI PRISM genetic analyzer (Thermo Fisher, Waltham, Massachusetts, USA) were used for direct sequencing according to the manufacturer's instructions. Because of the insufficient genomic DNA and failure of PCR reaction, not every donor had complete SNP data available.

Statistical Analysis

Single-locus association tests were performed to identify the donor SNPs that were associated with the defined outcomes in the recipients. The allele or genotype frequencies between cases (patients with the indicated outcomes) and controls (patients without the indicated outcomes) were compared with Cochran-Armitage Trend test (or Trend test) and the allelic test using the PLINK software v1.07 (31). The allele effects of the SNPs on each outcome were further examined using the logistic regression analysis assuming three modes of inheritance: additive model, recessive model, or dominant models. $p < 0.05$ was considered statistically significant. The Haploview 4.2 (32) software was used to determine the linkage disequilibrium (LD). The pairwise linkage disequilibrium value D' and the haplotype blocks of SNPs were determined. The haplotype blocks were defined as the SNPs in this region had no evidence for historical recombination.

RESULTS

Patient Characteristics and Study Design

A total of 163 patients receiving HSCT including 99 patients with AML and 64 patients with ALL were enrolled in this study. Clinical characteristics and the tracking data of mortality, CMV infection, relapse, and GVHD for these patients are listed in **Table 1**.

With the importance of co-stimulatory signals in the immune system and transplantation tolerance, the associations of the four co-stimulatory genes including CTLA4, TNFSF4, CD28, and PDCD1 with the mortality, relapse, CMV infection, and GVHD after HSCT were analyzed in this study. Based on our initial screening of SNPs in the CTLA4 gene of the Taiwanese population, the importance of promoter and exon 1 in gene expression, and the SNPs with clinical association, the genomic regions covering the promoter, exon 1, exon 4, and 3'-UTR of CTLA-4 (17 SNPs), the promoter and exon 1 of TNFSF4 (3 SNPs), the promoter and exon 1 of CD28 (9 SNPs), and the promoter, exon 1, and exons 4–5 including intron 4 of PDCD1 (5 SNPs) for all donors were amplified by PCR using the forward and reverse primers (**Tables 2**, **3**). The PCR amplicons were sequenced and the association of candidate SNPs with the adverse outcomes of patients with AML and ALL were analyzed by Trend test and allelic test, and by logistic regression analysis with additive, dominant, or recessive mode, respectively. The genotype and allele frequencies for all donors are summarized in the **Supplementary Tables S2–S5**.

Association of Donor SNPs With the Mortality, CMV Infection, and Relapse of Patients With AML and ALL

By analyzing a total of 34 SNPs, 4 SNPs (rs733618, rs11571316, and rs3087243 in CTLA-4, and rs41386349 in PDCD1) and 1 SNP (rs11571315) elicited significant recessive allelic effects and contributed to the post-HSCT mortality for patients with AML

TABLE 3 | Primer sequences for amplification of candidate SNPs.

Gene name	Genomic region	Primer sequence	PCR product (bp)
CTLA4	Promoter	F: 5'-GGCAACAGAGACCCCACCGTT-3'	1233
		R: 5'-GAGGACCTTCCTTAAATCTGGAGAG-3'	
	Promoter	F: 5'-CTCTCCAGATTTAAGGAAGGTCCTC-3'	1169
	and exon 1	R: 5'-GGAATACAGAGCCAGCCAAGCC-3'	
	Exon 4	F: 5'- CTAGGGACCCAATATGTGTTG-3'	1039
		R: 5'-AGAAACATCCCAGCTCTGTC-3'	
	Exon 4	F: 5'-GCTTGGAAACTGGATGAGGTCATAGC-3'	1204
	and 3'-UTR	R: 5'-AGAGGAAGAGACACAGACAGAGTTGC-3'	
TNFSF4	Promoter	F: 5'-GGCTTGGAGTCTATGATATTGTGCC-3'	1725
	and exon 1	R: 5'-GAAGGGCGTTTAACCACACTTTACG-3'	
CD28	Promoter	F: 5'- GGGTGGTAAGAATGTGGATGAATC-3'	1961
	and exon 1	R: 5'-CAAGGCATCCTGACTGCAGCA-3'	
PDCD1	Promoter	F: 5'-ACCCACACAGCCTCACATCTCT-3'	1778
	and exon 1	R: 5'-AAACTGAGGGTGGAAGGTCCCT-3'	
	Exon 4, intron 4	F: 5'-TGGTGACCCCAAGTGTGTTTCTC-3'	2234
	and exon 5	R: 5'-GAGGAATTTTTCACCGGAGGGC-3'	

F, forward primer; R, reversed primer.

and ALL, respectively (**Table 4**). For patients with AML, the C allele of rs733618 (CC vs. CT+TT, $p = 0.0376$, OR = 2.77, and 95% CI = 1.07–7.22) and the G allele of rs11571316 (GG vs. AA+AG, $p = 0.0441$, OR = 2.32, and 95% CI = 1.03–5.24) located in the promoter region of CTLA4 were associated with higher risk for mortality. The SNP rs3087243 in the 3'-UTR of CTLA4 (GG vs. AA+AG, $p = 0.0441$, OR = 2.32, and 95% CI = 1.03–5.24) and rs41386349 in the intron 4 of PDCD1 (GG vs. AA+AG, $p = 0.0362$, OR = 2.62, and 95% CI = 1.07–6.42) also conferred recessive effects to the risk for mortality of patients with AML. In addition, the SNP of rs41386349 also elicited additive effects on post-HSCT mortality for patients with AML. For patients with ALL, the C allele of rs11571315 (CC vs. CT+TT, $p = 0.0289$, OR = 6.14, 95% CI = 1.21–30.99) in the promoter region of CTLA4 was found to associate with higher risk for mortality.

One SNP (rs6705653 in PDCD1) and four SNPs (rs36084323, rs41386349, rs6705653, and rs2227982 in PDCD1) were found to associate with CMV infection in patients with AML and ALL, respectively (**Table 5**). For patients with AML, the C allele of rs6705653 (CC+CT vs. TT, $p = 0.0138$, OR = 7.91, and 95% CI = 1.54–40.71) elicited a dominant effect and contributed to the risk for CMV infection. For patients with ALL, the alternative T allele of the same SNP rs6705653 elicited an additive (Trend test: $p = 0.0198$, and additive effect: $p = 0.0186$) or a dominant effect to the risk for CMV infection (TT+CT vs. CC, $p = 0.0201$, OR = 3.95, and 95% CI = 1.25–12.49). The C allele of rs36084323, A allele of rs41386349, and G allele of rs2227982 in PDCD1 gene also associated with a higher risk for CMV infection (allele model: $p = 0.0265$, 0.0356, and 0.0252, respectively).

One SNP (rs200353921 in CD28) and three SNPs (rs5839828, rs36084323, and rs2227982 in PDCD1) were associated with the risk of disease relapse in patients with AML and ALL, respectively (**Table 6**). For patients with AML, the T allele of rs200353921 located on the promoter region of CD28 gene was associated with a higher risk of relapse (allele model $p = 0.0343$ for T vs. A, OR = 2.1, and 95% CI = 1.06–4.18). For patients with ALL, the G7 allele of rs5839828, the C allele of rs36084323, and the G allele of rs2227982 in the PDCD1 gene were also associated with a higher risk for disease relapse (allele model: $p = 0.0008$, 0.0095, and 0.0018, respectively).

Association of Donor SNPs With the Status of GVHD in Patients With AML and ALL

The status of GVHD was classified into four categories including GVHD III–IV (severe GVHD), GVHD I–II (mild GVHD), chronic GVHD (cGVHD), and no GVHD. Two SNPs (rs1234314 and rs45454293) in the promoter region of TNFSF4 were associated

TABLE 4 | The SNPs associated with the mortality post-HSCT.

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
AML											
rs733618	CTLA4 (-1722) promoter	C	C/C	C/T	T/T	0.1066	0.0757	1.69 (0.95-3.02)	Additive	0.1072	1.56 (0.91–2.66)
Case		52 (58.43)	18 (69.23)	16 (43.24)	14 (46.67)				Dominant	0.5106	1.34 (0.56–3.19)
Control		37 (41.57)	8 (30.77)	21 (56.76)	16 (53.33)				Recessive	0.0376	2.77 (1.07–7.22)
rs11571316	CTLA4 (-1577) promoter	G	A/A	A/G	G/G	0.0652	0.0706	1.82 (0.95-3.47)	Additive	0.0596	1.96 (0.98–3.95)
Case		79 (56.03)	2 (40)	17 (41.46)	31 (62)				Dominant	0.5821	1.67 (0.27–10.4)
Control		62 (43.97)	3 (60)	24 (58.54)	19 (38)				Recessive	0.0441	2.32 (1.03–5.24)
rs3087243	CTLA4 (ct60) 3'-UTR	G	A/A	A/G	G/G	0.0621	0.0706	1.82 (0.95-3.47)	Additive	0.0596	1.96 (0.98–3.95)
Case		79 (56.03)	2 (40)	17 (41.46)	31 (62)				Dominant	0.5821	1.67 (0.27–10.4)
Control		62 (43.97)	3 (60)	24 (58.54)	19 (38)				Recessive	0.0441	2.32 (1.03–5.24)
rs41386349	PDCD1 (IVS4+251) intron 4	G	A/A	A/G	G/G	0.0289	0.0201	2.45 (1.16-5.2)	Additive	0.0267	2.37 (1.11–5.08)
Case		77 (57.04)	1 (20)	11 (40.74)	33 (61.11)				Dominant	0.1713	4.76 (0.52–43.93)
Control		58 (42.96)	4 (80)	16 (59.26)	21 (38.89)				Recessive	0.0362	2.62 (1.07–6.42)
ALL											
rs11571315	CTLA4 (-1765) promoter	C	C/C	C/T	T/T	0.0833	0.0498	2.08 (1-4.31)	Additive	0.0678	1.95 (0.96–3.97)
Case		29 (60.42)	9 (81.82)	11 (42.31)	11 (42.31)				Dominant	0.3598	1.6 (0.59–4.39)
Control		19 (39.58)	2 (18.18)	15 (57.69)	15 (57.69)				Recessive	0.0289	6.14 (1.21–30.99)

TABLE 5 | The SNPs associated with CMV infection post-HSCT.

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
AML											
rs6705653	PDCD1 (IVS4+541) intron 4	C	C/C	C/T	T/T	0.0947	0.0633	1.92 (0.97–3.82)	Additive	0.0852	1.78 (0.93–3.44)
Case		83 (68.60)	31 (67.39)	21 (72.41)	2 (22.22)				Dominant	0.0138	7.91 (1.54–40.71)
Control		38 (31.40)	15 (32.61)	8 (27.59)	7 (77.78)				Recessive	0.5139	1.35 (0.55–3.29)
ALL											
rs36084323	PDCD1 (-606) promoter	C	C/C	C/T	T/T	0.054	0.0265	2.39 (1.11–5.15)	Additive	0.0413	2.2 (1.04–4.69)
Case		28 (51.85)	9 (60)	10 (41.67)	4 (23.53)				Dominant	0.0854	3.09 (0.86–11.08)
Control		26 (48.15)	6 (40)	14 (58.33)	13 (76.47)				Recessive	0.0874	2.89 (0.86–9.72)
rs41386349	PDCD1 (IVS4+251) intron 4	A	A/A	A/G	G/G	0.0609	0.0356	2.86 (1.08–7.56)	Additive	0.0515	2.64 (1–6.99)
Case		13 (61.90)	2 (66.67)	9 (60)	12 (31.58)				Dominant	0.0399	3.4 (1.06–10.89)
Control		8 (38.10)	1 (33.33)	6 (40)	26 (68.42)				Recessive	0.3752	3.05 (0.26–35.33)
rs6705653	PDCD1 (IVS4+541) intron 4	T	C/C	C/T	T/T	0.0198	0.0100	3.04 (1.31–7.04)	Additive	0.0186	2.81 (1.19–6.61)
Case		21 (61.76)	7 (26.92)	11 (55)	5 (71.43)				Dominant	0.0201	3.95 (1.25–12.49)
Control		13 (28.24)	19 (73.08)	9 (45)	2 (28.57)				Recessive	0.1269	3.89 (0.69–22.03)
rs2227982	PDCD1 (+699) exon 5	G	A/A	A/G	G/G	0.0534	0.0252	2.53 (1.13–5.67)	Additive	0.0541	2.06 (0.99–4.27)
Case		30 (57.69)	4 (26.67)	8 (47.06)	11 (61.11)				Dominant	0.0797	3.27 (0.88–12.19)
Control		23 (42.31)	11 (73.33)	9 (52.94)	7 (38.89)				Recessive	0.1120	2.62 (0.8–8.54)

with the risk for severe GVHD (**Table 7**) in patients with AML (rs1234314: Trend test, $p = 0.006$; allele model, $p = 0.0114$ for C vs. G, OR = 7.39, and 95% CI = 1.58–34.52; and rs45454293: Trend test, $p = 0.0145$, allele model $p = 0.0100$ for T vs. C, OR = 4.86, and 95% CI = 1.47–16.07). Both SNPs elicited additive and recessive effects on the risk for severe GVHD. No SNP was found to associate with the risk for severe GVHD in patients with ALL.

No SNP was associated with the risk for mild GVHD in patients with AML. Four SNPs (rs231775 in CTLA4, and rs41386349, rs6705653, and rs2227982 in PDCD1) were associated with the risk for mild GVHD (GVHD I–II) in patients with ALL. The A allele of rs231775 on exon 1 of CTLA4 (allele model: $p = 0.0343$ for A vs. G, OR = 2.28, and 95% CI = 1.07–4.89), the A allele of rs41386349 (allele model: $p = 0.0436$ for A vs. G, OR = 2.71, and 95% CI = 1.03–7.1), the T allele of rs6705653 (Trend test: $p = 0.0086$; allele model: $p = 0.0039$ for T vs. C, OR = 3.53, and 95% CI = 1.51–8.28) in the intron 4 of PDCD1, and the G allele of rs2227982 (Trend test: $p = 0.0194$; allele model: $p = 0.0055$ for G vs. A, OR = 3.4, and 95% CI = 1.44–8.03) in the exon 5 of PDCD1 gene contributed to a higher risk for mild GVHD. The three SNPs in the PDCD1 gene were also associated with CMV infection as above mentioned.

No SNP was found to associate with the risk for cGVHD in patients with AML. Five SNPs (rs5742909 and rs231775 in CTLA4, rs28541784 in CD28, and rs6705653 and rs2227982 in PDCD1) were associated with the risk for cGVHD in patients with ALL. The C allele of rs5742909 (allele model: $p = 0.0465$ for C vs. T) and the G allele of rs231775 (recessive model: $p = 0.0279$ for GG vs. AA+AG) on the CTLA4 gene contributed to the higher risk for cGVHD, yet in different modes. In addition, the T allele of rs28541784 on CD28 gene was associated with a higher risk for cGVHD (Trend test: $p = 0.0473$; allele model: $p = 0.0303$ for T vs. C, OR = 2.78, and 95% CI = 1.11–6.98). Of the SNPs located in the PDCD1 gene, the C allele of rs6705653 (allele model: $p = 0.0066$ for C vs. T) and the A allele of rs2227982 (allele model: $p = 0.0305$ for A vs. G) also contributed to the higher risk for cGVHD.

Two SNPs (rs3181096 and rs3181098 in CD28) and four SNPs (rs4553808, rs62182595, rs16840252, and rs5742909 in CTLA4) were associated with the protective effects on the development of GVHD in patients with AML and ALL, respectively. The dominant effects of the T allele of rs3181096 and the A allele of rs3181098 in the promoter region of CD28 conferred protective effects on the development of GVHD in patients with AML (dominant model: $p = 0.0231$ and 0.0235, respectively). The G allele of rs4553808 (allele model: $p = 0.0452$ for G vs. A, OR = 4.63, and 95% CI = 1.04–20.58), the A allele of rs62182595 (allele model: $p = 0.0330$ for A vs. G, OR = 5.19, and 95% CI = 1.15–23.4), the T allele of rs16840252 (allele model: $p = 0.0350$ for T vs. C, OR = 5.1, and 95%

TABLE 6 | The SNPs associated with disease relapse post-HSCT.

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
AML											
rs200353921	CD28 (–879) promoter	T	A/A	A/T	T/T	0.1437	0.0343	2.1 (1.06–4.18)	Additive	0.1187	1.5 (0.9–2.5)
Case		58 (48.33)	7 (30.43)	2 (33.33)	28 (49.12)				Dominant	0.1586	2.08 (0.76–5.71)
Control		62 (51.67)	16 (69.57)	4 (66.67)	29 (50.88)				Recessive	0.1125	2.15 (0.84–5.48)
ALL											
rs5839828	PDCD1 (–763) promoter	G7	G6	G6/G7	G7	0.0011	0.0008	3.8 (1.75–8.48)	Additive	0.0024	4.02 (1.64–9.83)
Case		27 (56.25)	3 (14.29)	11 (42.31)	8 (72.73)				Dominant	0.0088	6.33 (1.6–25.04)
Control		21 (43.75)	18 (85.71)	15 (57.69)	3 (27.27)				Recessive	0.0140	6.29 (1.46–27.05)
rs36084323	PDCD1 (–606) promoter	C	C/C	C/T	T/T	0.0206	0.0095	2.8 (1.29–6.17)	Additive	0.0179	2.6 (1.18–5.7)
Case		28 (51.85)	9 (60)	10 (41.67)	3 (17.65)				Dominant	0.0366	4.43 (1.11–17.78)
Control		26 (48.15)	6 (40)	14 (58.33)	14 (82.35)				Recessive	0.0606	3.23 (0.96–10.92)
rs2227982	PDCD1 (+699) exon 5	G	A/A	A/G	G/G	0.007	0.0018	3.9 (1.67–9.35)	Additive	0.0083	3.01 (1.33–6.8)
Case		29 (54.72)	3 (20)	5 (29.41)	12 (66.67)				Dominant	0.0682	3.78 (0.91–15.64)
Control		24 (45.28)	12 (80)	12 (70.59)	6 (33.33)				Recessive	0.0055	6 (1.7–21.13)

TABLE 7 | The SNPs associated with GVHD post-HSCT.

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
GVHD III–IV AML											
rs1234314	TNFSF4 (–738) promoter	C	C/C	C/G	G/G	0.006	0.0114	7.39 (1.58–34.52)	Additive	0.0148	7.19 (1.48–34.86)
Case		10 (12.99)	4 (22.22)	2 (4.88)	0 (0)				Dominant	0.9475	n/a
Control		67 (81.01)	14 (77.78)	39 (95.12)	30 (100)				Recessive	0.0123	9.86 (1.66–58.62)
rs45454293	TNFSF4 (–582) promoter	T	C/C	C/T	T/T	0.0145	0.0100	4.86 (1.47–16.07)	Additive	0.0162	4.77 (1.34–16.96)
Case		6 (17.65)	2 (3.45)	2 (7.69)	2 (50)				Dominant	0.1039	4.31 (0.75–24.81)
Control		28 (82.35)	56 (96.55)	24 (92.31)	2 (50)				Recessive	0.0077	20 (2.24–178.9)
GVHD I–II ALL											
rs231775	CTLA4 (+49) exon 1	A	A/A	A/G	G/G	0.0719	0.0343	2.28 (1.07–4.89)	Additive	0.0543	2.05 (0.99–4.26)
Case		22 (45.83)	5 (41.66)	12 (50)	4 (16)				Dominant	0.0156	4.7 (1.35–16.35)
Control		26 (54.17)	7 (58.34)	12 (50)	21 (84)				Recessive	0.5572	1.47 (0.41–5.34)
rs41386349	PDCD1 (IVS4+251) intron 4	A	A/A	A/G	G/G	0.0604	0.0436	2.71 (1.03–7.1)	Additive	0.0613	2.51 (0.96–6.53)
Case		12 (57.14)	3 (100)	6 (40)	12 (25)				Dominant	0.1874	2.17 (0.69–6.8)
Control		9 (42.86)	0 (0)	9 (60)	26 (75)				Recessive	0.9754	n/a
rs6705653	PDCD1 (IVS4+541) intron 4	T	C/C	C/T	T/T	0.0086	0.0039	3.53 (1.51–8.28)	Additive	0.0091	3.29 (1.35–8.02)

(Continued)

TABLE 7 | Continued

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
Case		19 (55.88)	5 (19.23)	9 (45)	5 (71.43)				Dominant	0.0165	4.52 (1.33–15.43)
Control		15 (44.12)	21 (80.77)	11 (55)	2 (28.57)				Recessive	0.0517	5.71 (1–32.79)
rs2227982	PDCD1 (+699) exon 5	G	A/A	A/G	G/G	0.0194	0.0055	3.4 (1.44–8.03)	Additive	0.0178	2.63 (1.19–5.85)
Case		27 (50.94)	2 (13.33)	7 (41.18)	10 (55.56)				Dominant	0.0291	6.14 (1.21–31.06)
Control		26 (49.06)	13 (86.67)	10 (58.82)	8 (44.44)				Recessive	0.0595	3.19 (0.96–10.62)
Chronic GVHD ALL											
rs5742909	CTLA4 (–319) promoter	C	C/C	C/T	T/T	0.0509	0.0465	3.91 (1.03–14.86)	Additive	0.0541	3.82 (0.98–14.87)
Case		61 (53.98)	29 (56.86)	3 (27.27)	0 (0)				Dominant	0.9856	n/a
Control		52 (46.02)	22 (43.14)	8 (72.73)	1 (100)				Recessive	0.0576	3.95 (0.96–16.23)
rs231775	CTLA4 (+49) exon 1	G	A/A	A/G	G/G	0.1692	0.1049	1.84 (0.88–3.82)	Additive	0.1377	1.69 (0.85–3.37)
Case		42 (56.76)	6 (50)	8 (33.33)	17 (68)				Dominant	0.9495	1.04 (0.3–3.66)
Control		32 (43.24)	6 (50)	16 (66.67)	8 (32)				Recessive	0.0279	3.34 (1.15–9.73)
rs28541784	CD28 (–891) promoter	T	C/C	C/T	T/T	0.0473	0.0303	2.78 (1.11–6.98)	Additive	0.0495	2.46 (1.01–6.02)
Case		19 (70.37)	15 (41.67)	11 (64.71)	4 (80)				Dominant	0.0536	3 (0.99–9.1)
Control		8 (29.63)	21 (58.33)	6 (35.29)	1 (20)				Recessive	0.2161	4.15 (0.44–39.21)
rs6705653	PDCD1 (IVS4+541) intron 4	C	C/C	C/T	T/T	0.0114	0.0066	3.36 (1.41–8.02)	Additive	0.0131	3.12 (1.28–7.63)
Case		42 (58.33)	17 (65.38)	8 (40)	1 (14.29)				Dominant	0.0792	7.14 (0.8–63.38)
Control		30 (41.67)	9 (34.62)	12 (60)	6 (85.71)				Recessive	0.0220	3.78 (1.22–11.71)
rs2227982	PDCD1 (+699) exon 5	A	A/A	A/G	G/G	0.0795	0.0305	2.43 (1.09–5.42)	Additive	0.0624	1.99 (0.97–4.1)
Case		28 (59.57)	10 (66.67)	8 (47.06)	6 (33.33)				Dominant	0.1240	2.57 (0.78–8.51)
Control		19 (40.43)	5 (33.33)	9 (52.94)	12 (66.67)				Recessive	0.0897	3 (0.85–10.6)
No GVHD AML											
rs3181096	CD28 (–1328) promoter	T	C/C	C/T	T/T	0.0849	0.0682	2.66 (0.93–7.58)	Additive	0.0844	2.47 (0.89–6.88)
Case		7 (15.56)	1 (1.85)	7 (22.58)	0 (0)				Dominant	0.0231	11.97 (1.42–100.74)
Control		38 (84.44)	53 (98.15)	24 (77.42)	7 (100)				Recessive	0.9722	n/a
rs3181098	CD28 (–1042) promoter	A	A/A	A/G	G/G	0.0889	0.0723	2.63 (0.92–7.5)	Additive	0.0898	2.43 (0.88–6.75)
Case		7 (15.56)	0 (0)	7 (23.33)	1 (1.92)				Dominant	0.0235	11.9 (1.41–100.34)
Control		38 (84.44)	7 (100)	23 (76.67)	51 (98.08)				Recessive	0.9717	n/a
ALL											
rs4553808	CTLA4 (–1661) promoter	G	A/A	A/G	G/G	0.0747	0.0452	4.63 (1.04–20.58)	Additive	0.0627	4.42 (0.93–21.01)
Case		3 (23.77)	3 (5.77)	1 (9.09)	1 (100)				Dominant	0.2255	3.27 (0.49–21.94)

(Continued)

TABLE 7 | Continued

SNP	Gene position	Risk allele no. (%)	No. of patients (%)			Trend *p*	Allele *p*	OR	Model	Logistic regression *p*	OR
Control		10 (76.23)	49 (94.23)	10 (90.91)	0 (0)				Recessive	0.9957	n/a
rs62182595	CTLA4 (–1478) promoter	A	A/A	A/G	G/G	0.0599	0.0330	5.19 (1.15–23.4)	Additive	0.0515	4.77 (1–22.83)
Case		3 (25)	1 (100)	1 (10)	3 (5.66)				Dominant	0.1824	3.7 (0.55–25.14)
Control		9 (75)	0 (0)	9 (90)	50 (94.34)				Recessive	0.9957	n/a
rs16840252	CTLA4 (–1147) promoter	T	C/C	C/T	T/T	0.0678	0.0350	5.1 (1.13–22.98)	Additive	0.0538	4.7 (0.98–22.49)
Case		3 (25)	3 (5.77)	1 (10)	1 (100)				Dominant	0.1894	3.63 (0.53–24.64)
Control		9 (75)	49 (94.23)	9 (90)	0 (0)				Recessive	0.9953	n/a
rs5742909	CTLA4 (–319) promoter	T	C/C	C/T	T/T	0.0722	0.0479	4.54 (1.02–20.2)	Additive	0.0655	4.36 (0.92–20.69)
Case		3 (23.77)	3 (5.88)	1 (9.09)	1 (100)				Dominant	0.2337	3.2 (0.48–21.5)
Control		10 (76.23)	48 (94.12)	10 (90.91)	0 (0)				Recessive	0.9953	n/a

CI = 1.13–22.98), and the T allele of rs5742909 (allele model: p = 0.0479 for T vs. C, OR = 4.54, and 95% CI = 1.02–20.2) of the CTLA4 gene conferred better protective effects on the development of GVHD in patients with ALL.

Linkage Disequilibrium

The SNPs (n = 20) that were associated with the risk for adverse outcomes in patients with either AML or ALL were subject to LD analysis (**Figure 1**). Several pairs of SNPs had high or complete LD including the rs3087243 with rs231775 (D' = 0.97), with rs62182595 (D' = 0.98), and with rs11571316 (D' = 0.96) in the CTLA4 gene; the rs6705653 with rs41386349 (D' = 1) in the PDCD1 gene; and the rs3181096 with rs3181098 (D' = 0.96) in the CD28 gene. In addition, there were three haplotype blocks including the SNPs in the CD28, CTLA4 and PDCD1, respectively. These data imply a potential genetic linkage of these SNPs in the human genome.

DISCUSSION

The SNPs located in the HLA regions have been reported to associate with the post-HSCT adverse outcomes (33). In this study, we investigated further whether these is an association between 34 donor SNPs in the four co-stimulatory genes (TNFSF4, CTLA4, CD28, and PDCD1) and the occurrence of adverse outcomes (mortality, relapse, CMV infection, and GVHD) for patients with AML and ALL. Our data revealed that 10 and 12 SNPs located in these four genes were related to the adverse outcomes of patients with AML and ALL, respectively.

Co-stimulatory molecules play a critical role in immune regulation and are involved in the pathogenesis of autoimmune diseases, cancers, and graft rejection (34). During T-cell activation, CD28 provides a stimulatory signal when it interacts with CD80/CD86 on the antigen-presenting cells. CTLA4 is then expressed on the activated T cell, playing a role in negative regulation of T-cell activation by competing with CD28 for CD80/CD86 to prevent excessive T-cell activation (35, 36). PDCD1, like CTLA4, plays a negative regulatory role in T-cell activation to develop immune tolerance, which can prevent the development of autoimmune diseases or prevent the immune system from killing cancer cells (37). In addition, the OX40 ligand encoded by TNFSF4 is the key to coordinate innate and adaptive immune cells and plays an important role in the life cycle of immune cells, such as differentiation, activation, inhibition, and apoptosis (38).

Several findings were noted in this study. Most adverse outcomes-related SNPs are unique to patients with AML and ALL, except rs6705653, which is associated with CMV infection for both leukemic types. These data imply that the four co-stimulatory molecules may elicit various functional activity toward AML and ALL cancer cells. In addition, several SNPs are related to more than one clinical outcome in patients with ALL. The SNP of rs41386349 is related to the risk for CMV infection and GVHD I–II, rs36084323 is related to CMV infection and relapse, rs6705653 is related to CMV infection, GVHD I–II, and chronic GVHD, and rs2227982 is related to CMV infection, relapse, GVHD I–II, and chronic GVHD for patients with ALL. These data further indicate that the co-stimulatory molecules are involved in multiple aspects of immune activity and susceptibility of CMV infection in transplantation.

Notably, the SNPs of CTLA4 and PDCD1 are associated with several adverse outcomes in patients with AML or ALL. Four SNPs in the CTLA4 gene are associated with the risk for mortality (rs733618, rs11571316, rs3087243, and rs11571315). These SNPs are also known to associate with autoimmune diseases and cancers (39). Another five SNPs in the CTLA4 gene are related to the status of GVHD (rs5742909, rs4553808, rs62182595, rs16840252, and rs231775). Among these SNPs, rs733618, rs11571315, rs11571316, rs5742909, rs4553808, rs62182595, and rs16840252 are within the promoter region, rs231775 is in exon 1, and rs3087243 is in the 3'-UTR of CTLA4 gene. Because CTLA4 expression is important to

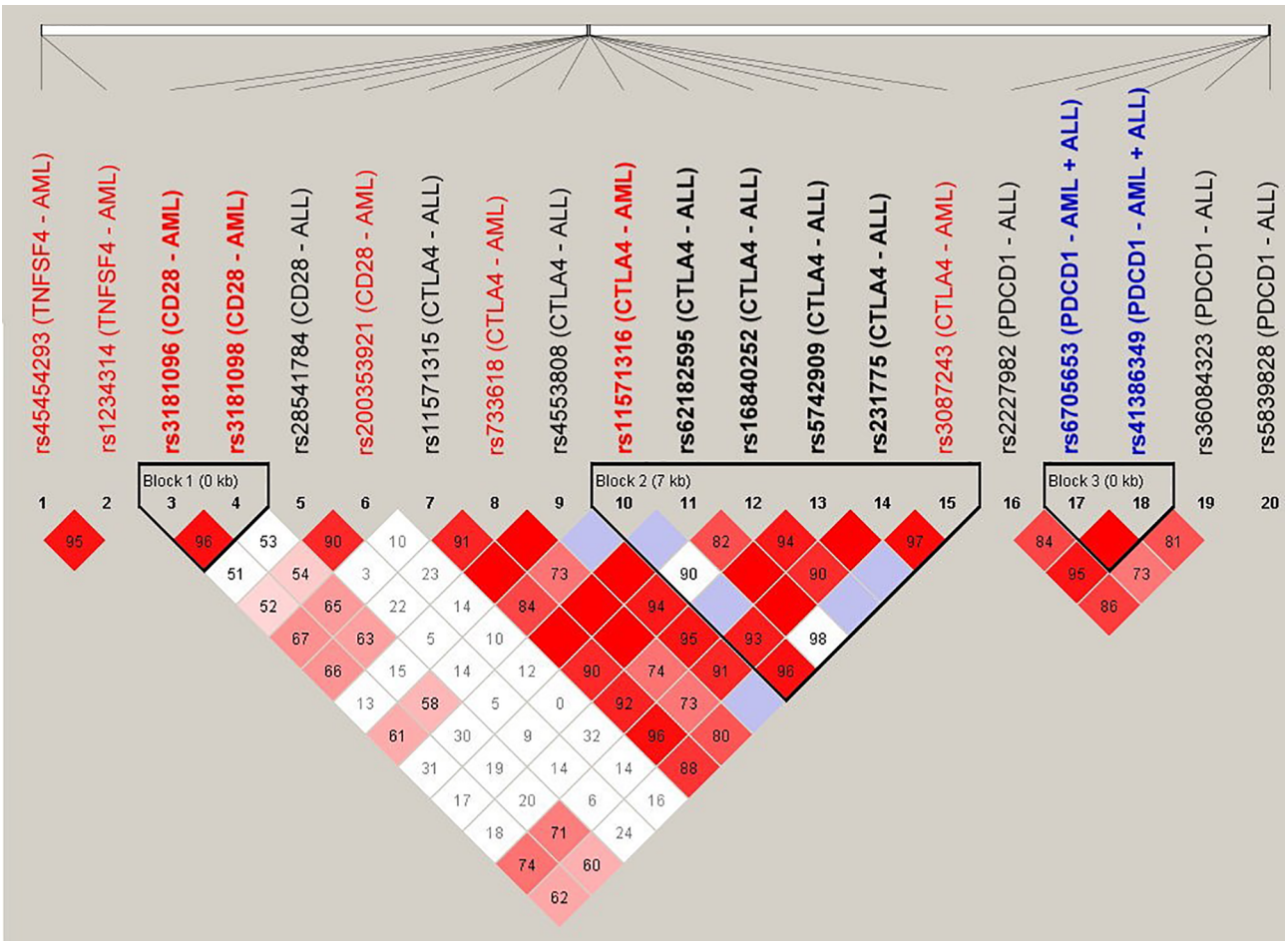

FIGURE 1 | Linkage disequilibrium (LD) analysis of the donor SNPs that were associated with the adverse outcomes of patients with AML and ALL. The pairwise linkage disequilibrium (D') was given for each pair of SNPs. The red boxes indicated that the pairs of SNPs had high LD, and the lighter the color, the smaller the LD was.

evade surveillance from host immune cells (40), these SNPs are likely to modulate CTLA4 gene expression, thereby altering the immune response and conferring a risk for mortality post-HSCT. Consistent with this notion, the SNPs located on the promoter region have been shown to elicit effects on gene expression (41), The genotypic variants of rs3087243 have been shown to associate with CTLA4 expression in patients with inflammatory bowel disease (42). The A allele of rs231775 is known to produce higher mRNA efficiency than the G allele, leading to produce more CTLA4 protein (30). It is worthy to investigate further whether the abovementioned SNPs regulate CTLA4 expression leading to aGVHD and cGVHD.

Five SNPs (rs36084323, rs5839828, rs41386349, rs6705653, and rs2227982) in PDCD1 gene are associated with the risk for relapse, mortality, CMV infection, and GVHD. This is consistent with the key roles of PDCD1 in regulating allogeneic immune response in transplantation. Maintenance of graft tolerance is related to the interaction between PDCD1 (PD-1) and PD-L (43). Post-transplantation lymphoproliferative disorder, which was developed under the condition of T-cell dysfunction or immunosuppression after HSCT, is also related to the expression of PDCD1 (44). Among the SNPs, rs5839828 and rs36084323 are within the promoter region, rs6705653 and rs41386349 are in intron 4, and rs2227982 is in exon 5. The SNPs located in the promoter and exon regions may affect the expression of transcription and the alteration of coding amino acid, respectively. Whether intronic SNP has any effect on PDCD1 expression is not clear. Nevertheless, aberrant splicing has been linked to the intronic SNP and causes protein mutation (45).

Four SNPs (rs200353921, rs3181096, rs3181098, and rs28541784) in CD28 gene are associated with the GVHD grades and relapse for patients with AML and ALL. These SNPs may directly or indirectly alter CD28 expression to induce different degrees of cellular responses (46), which, in turn, affect the risk of GVHD and relapse for leukemic patients after HSCT. The interplay between CD28 and GVHD has been reported in several previous studies. CD28 in donor T cells contributes to the pathogenesis and severity of GVHD in a mouse model (17). Abnormal expression of CD28 and CTLA4 in peripheral blood leukocytes of patients with AML may promote the development of aGVHD after HSCT (47). Consistent with these previous studies, our data revealed that the

donor SNPs in CD28 gene were related to the development of GVHD in AML patients, regardless of the grade status. Moreover, two SNPs (rs45454293 and rs1234314) in the promoter region of TNFSF4 gene are associated with the development of GVHD grades III and IV for patients with AML. In accord with our findings, Tripathi et al. showed that OX40L (TNFSF4)–OX40 interaction not only induces aGVHD, but also is an essential part in the progression of aGVHD (48). The SNPs in the promoter region is likely to modulate OX40L (TNFSF4) expression, resulting in excessive OX40L–OX40 interaction, which subsequently increases the risk of GVHD.

In addition to genetic studies to associate SNPs with the prognosis of leukemia patients post-HSCT, studies have been reported to integrate both clinical variables and genetic variables in generating predictive model for clinical outcomes after HSCT (49–51). In this regard, Martinez-Laperche et al. applied a complex estimation method, the least absolute shrinkage and selection operation (LASSO) procedure, to generate a predictive model to improve the prediction of severe GVHD (grades III–IV) (49). The model including both clinical variables and genetic variables is better than the models containing only clinical variables or only genetic polymorphisms. Another risk model integrating SNPs and clinical variables have also been demonstrated to predict the risk for GVHD in specific organs (50, 51). An extension of the current study is to integrate clinical variables with our SNP data for multivariate regression analysis and association study. Increasing the enrollment number of donor–recipient pairs may further validate and confirm the importance of these SNPs in the development of adverse outcomes post-HSCT for patients with leukemia.

In conclusion, a total of 10 and 12 SNPs in the co-stimulatory genes are associated with the post-HSCT adverse outcomes for patients with AML and ALL, respectively. Because these SNPs are present in the donor DNA, it provides a basis for developing a screening panel of SNPs to search and select appropriate donors for transplantation. It is also worthy to investigate further the effects of these SNPs on the expression of these co-stimulatory genes to elucidate the underlying mechanisms of transplantation failure.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Chang Gung Memorial Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

D-PC conceived and designed the experiments. P-NW provided laboratory samples. D-PC, W-TL, and C-PT wrote the draft of the manuscript. F-PH performed the experiments and analyzed the data. W-TW reviewed and approved the final draft. S-WC and C-PT reviewed literature, and analyzed and interpreted data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.730507/full#supplementary-material

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