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Differential effects of Belatacept on virus-specific memory *versus de novo* allo-specific T cell responses of kidney transplant recipients and healthy donors

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ABSTRACT

Belatacept, Nulojix®, inhibits the interaction of CD28 on naïve T cells with B7.1/B7.2 (CD80/86) on antigen presenting cells, leading to T cell hyporesponsiveness and anergy and is approved as immunosuppressive drug in kidney transplantation. Due to its specificity for B7.1/2 molecules, side effects are reduced compared to other immunosuppressive drugs like calcineurin- and mTOR-inhibitors. Kidney transplant recipients under Belatacept-based immunosuppression presented with superior renal function and similar graft survival seven years after transplantation compared to cyclosporine treatment. However, *de novo* Belatacept-based immunosuppression was associated with increased risk of early rejections and viral (EBV) infections in clinical trials, especially in EBV-naïve patients. Since there is no vaccination against EBV infection available, EBV-derived virus like particles (EBV-VLPs) are currently developed as vaccine strategy. Here, we investigated the immunosuppressive effects of Belatacept compared to calcineurin- and mTOR inhibitors on allo- *versus* virus-specific T cells and the potency of EBV-VLPs to induce virus-specific T cell responses *in vitro*. Using PBMC of kidney recipients and healthy donors, we could demonstrate selective inhibition of allo-specific *de novo* T cell responses but not virus-specific memory T cell responses by Belatacept, as measured by IFN-γ production. In contrast, calcineurin inhibitors suppressed IFN-γ production of virus-specific memory $CD8^+$ T cells completely. These results experimentally confirm the concept that Belatacept blocks CD28-mediated costimulation in newly primed naïve T cells but does not interfere with memory T cell responses being already independent from CD28-mediated costimulation. Additionally, we could show that EBV-VLPs induce a significant though weak IFN-γ-mediated T cell response *in vitro* in both kidney recipients and healthy donors. In summary, we demonstrated that immunosuppression of kidney recipients by Belatacept may primarily suppress *de novo* allo-specific T cell responses sparing virus-specific memory T cells. Moreover, EBV-VLPs could represent a novel strategy for vaccination of immunocompromised renal transplant recipients to prevent EBV reactivation especially under Belatacept-based immunosuppression.

Abbreviations: CEF, CMV-EBV- and influenza peptides; CMV, cytomegalovirus; CNI, calcineurin inhibitor; CsA, cyclosporin A; DMSO, dimethylsulfoxide; EBV, Epstein-Barr virus; Ever, everolimus; EV, extracellular vesicle; Grm, granzyme; HD, healthy donor; IFN-γ, interferon-γ; IL, interleukin; KTx, kidney transplantation; MMF, mycophenolate mofetil; mTORI, mammalian target of rapamycin inhibitor; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; Sir, sirolimus; Tac, tacrolimus; VLP, virus like particle

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1. Introduction

Kidney transplantation is one treatment option for patients with end-stage renal disease and dialysis-dependent insufficiency. Despite the fact that kidney donors and recipients are matched for most of their human leukocyte antigens (HLA-A, -B, −DR), acute or chronic graft rejection represent clinically relevant complications for kidney transplanted (KTx) patients. To prevent graft rejection, kidney recipients have to take immunosuppressive drugs interfering with different molecular mechanisms of immune activation. Clinically approved immunosuppressive drugs in kidney transplantation are calcineurin inhibitors (CNI) like cyclosporin A (CsA) and tacrolimus (Tac), which inhibit the phosphatase calcineurin [1]. Hence, CNI act mainly on T and NK cells by suppressing NFAT-dependent proliferation and cytokine production, *i.e.* interleukin (IL)-2 and interferon-γ (IFN-γ) in healthy individuals as well as in KTx patients [2–4]. Unfortunately CNI, particularly CsA, have nephrotoxic side effects [5], generating the clinical need to replace CNI by other immunosuppressive drugs with less nephrotoxicity. A promising alternative and yet more specific immunosuppressive mechanism is T-cell costimulation blockade by Belatacept, a fusion protein of the extracellular domain of CTLA-4 (cytotoxic T lymphocyte associated protein 4, CD152) with the Fc-part of IgG4 [6]. The interaction between CD28, expressed on naïve T cells, and B7.1/2 (CD80/CD86) on antigen presenting cells is the major costimulation pathway for complete T cell activation. T cell activation is tightly controlled by upregulation of the inhibitory receptor CTLA-4, which binds to B7.1/2 with higher affinity than CD28 and, therefore, costimulation blockade with Belatacept is based on this regulatory process [7]. Belatacept specifically interferes with T cell costimulation by binding with higher affinity to B7.1/2 due to two amino acid substitutions, thereby, blocking the interaction with CD28 on naïve T cells. Based on this specific mechanism, Belatacept should have fewer side effects while inhibiting graft rejection and loss in KTx patients as confirmed by the excellent clinical follow up data of the BENEFIT trial patients who presented improved glomerular filtration rates and reduced kidney toxicity [8]. One advantage of Belatacept-based immunosuppression may be related to the stage-specific dependence on costimulation since blocking of CD28 in naïve T cells leads to apoptosis or anergy [9]. In contrast, memory T cells have lost CD28 expression following repeated activation and, hence, are activated primarily *via* their TCR signal independently from CD28 [10,11]. This CD28 biology implies that immunosuppression *via* costimulation blockade in KTx patients may rather suppress *de novo* naïve T cell alloreactivity but maintain memory T cell responses directed against pathogens. However, experimental proof for this selective effect on naïve rather than memory T cells has not been demonstrated in KTx patients.

A second risk besides graft rejection for KTx patients under immunosuppression is infection or reactivation of latent viruses like cytomegalovirus (CMV) or Epstein-Barr virus (EBV), especially in high-risk constellations of virus-negative recipients receiving grafts from virus-positive donors. To date, there is no EBV-vaccine available in the clinical setting that could prevent EBV infection or reactivation in immunocompromised patients. The group of Hammerschmidt and Zeidler could proof functionality of virus like particles (VLP) carrying immunogenic proteins of EBV (EBV-VLPs) in terms of eliciting protective T and B cell responses *in vitro* and in *in vivo* mouse models [12]. In the context of kidney transplantation, information regarding T cell responses to these EBV-VLPs is currently not available. Therefore, we quantified the EBV-VLP-specific T cell responses of healthy donors and kidney recipients in order to determine the T cell immunity in EBV-positive donors against this EBV vaccine candidate. Here, we demonstrate i) differential suppressive effects of Belatacept only on *de novo* $CD8^+$ T cell responses, and ii) the potential of EBV-VLPs to elicit T cell responses even in immunosuppressed kidney recipients. Thus, our study contributes to a better understanding of the fine tuning of immunosuppression in kidney transplant recipients and supports the application of VLPs as novel EBV-vaccine candidate.

1.1. Objective

The aim of our study was to investigate the selective ability of Belatacept to suppress *de novo* allo-specific T cell responses in healthy donors and kidney transplanted patients *in vitro*, while preserving $CD8^+$ virus-specific memory T cell responses. Additionally, we tested whether EBV-VLPs could elicit specific T cell responses in both healthy donors and immunosuppressed KTx patients and might, therefore, represent a potential prophylactic EBV-vaccine for immunocompromised patients.

2. Materials and methods

2.1. Patients and sample collection

The Ethics Committee of Hannover Medical School approved the collection of blood from healthy donors (MHH ethics committee No. 968–2011) as well as from kidney transplanted patients (No. 5970.124). PBMC were prepared by Ficoll (Biochrom, Berlin, Germany) separation and cryopreserved. Demographic data of patients ($n = 12$) and healthy donors ($n = 8$) are given in Table 1. Induction therapy using the IL-2 receptor antagonist basiliximab was given to five KTx patients (41.67%), three (25%) patients had no induction therapy, two (16.67%) were treated with anti-thymocyte Ig (ATG) and two (16.67%) with rituximab. Standard immunosuppression comprised oral triple immunosuppression with CsA or Tac with corticosteroids and the nucleosid analogon MMF (mycophenolate-mofetil). One patient received Tac plus mTOR inhibitor (sirolimus; Tac/Sir). KTx patients and healthy donors were selected for ELISpot assays according to their HLA class I typing and presentation of CEF (CMV, EBV, Flu) peptides (Table 2).

2.2. Quantification of interferon-γ secreting T cells specific for viral peptides and VLPs by ELISpot

The anti-human IFN-γ antibody pair (BD Bioscience, San Jose, CA, USA) was used for ELISpot assays according to the manufacturer's instructions. To determine virus-specific $CD8^+$ T cells, PBMC of KTx or healthy individuals were stimulated with a pool of 23 CMV-, EBV- and influenza-specific peptides (CEF-peptides, Mabtech, Nacka Strand, Sweden). Allogeneic T cell responses were detected by stimulation with allogeneic EBV-transformed B cell lines generated from four different

Table 1
Demographic data of KTx patients and healthy donors.

KTx patients ($n = 12$)		Absolute numbers (%)	Mean trough levels
Sex	Male	8 (66.67%)	
	Female	4 (33.33%)	
Immunosuppression	Cyclosporin A	2 (16.67%)	30 ng/mL
	Tacrolimus	8 (66.67%)	9 +/− 2.45 ng/mL
	Tac/Sir	1 (8.33%)	6 ng/mL
	Sirolimus	1 (8.33%)	
Age		14–68 years	Median: 39 years
Viral status	EBV	9 (75%)	
	CMV	9 (75%)	
Healthy donors (n = 7)		Absolute numbers (%)	
Sex	Male	2 (28.57%)	
	Female	5 (71.43%)	
Age		29–55 years	Median: 44 years
Viral status	EBV	7 (100%)	
	CMV	0 (%)	

Table 2
HLA alleles of KTx patients and healthy donors with corresponding CEF-peptides

KTx#	HLA-Typing	Virus	corresponding CEF-peptide
#25	HLA-A3	influenza A	NP (265-273)
		EBV	BRLF1 (148-156)
		EBV	EBNA3A (603-611)
	HLA-A24	EBV	BRLF1 (28-37)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
#47	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-B44	EBV	EBNA3C (281-290)
		HCMV	pp65 (511-525)
#50	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-A3	influenza A	NP (265-273)
		EBV	BRLF1 (148-156)
		EBV	EBNA3A (603-611)
	HLA-B35	EBV	EBNA3A (458-466)
#51	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-A3	influenza A	NP (265-273)
		EBV	BRLF1 (148-156)
		EBV	EBNA3A (603-611)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
#62	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-A11	EBV	EBNA3B (416-424)
		EBV	BRLF1 (134-143)
#63	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
#79	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-A3	influenza A	NP (265-273)
		EBV	BRLF1 (148-156)
		EBV	EBNA3A (603-611)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
#93	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)

HD#	HLA-Typing	Virus	corresponding CEF-peptide
#1	HLA-A24	EBV	BRLF1 (28-37)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
	HLA-B27	EBV	EBNA3C (258-266)
		influenza A	NP (383-391)
#2	HLA-A1	influenza A	PB1 (591-599)
		influenza A	NP (44-52)
	HLA-A68	influenza A	NP (91-99)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
	HLA-B8	EBV	EBNA3A (158-166)
		EBV	EBNA3A (325-333)
		EBV	BZLF1 (190-197)
#3	HLA-A1	influenza A	PB1 (591-599)
		influenza A	NP (44-52)
	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-B7	HCMV	pp65 (415-429)
		EBV	EBNA3A (379-387)
	HLA-B8	EBV	EBNA3A (158-166)
		EBV	EBNA3A (325-333)
		EBV	BZLF1 (190-197)
#4	HLA-A1	influenza A	PB1 (591-599)
		influenza A	NP (44-52)
	HLA-A2	EBV	BMLF1 (259-267)
		influenza A	Matrix 1 (58-66)
		HCMV	pp65 (495-503)
	HLA-B8	EBV	EBNA3A (158-166)
		EBV	EBNA3A (325-333)
		EBV	BZLF1 (190-197)

donors (see Table S1 for information on HLA class I/II alleles). All stimulations were performed in triplicates for 18 h in RPMI1640, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin and 10% FCS (all Gibco Life Technologies, Darmstadt, Germany) without or with indicated inhibitors: Belatacept (Bristol-Meyer Squibb, Munich, Germany) or CD6-Ig (R&D Systems, Minneapolis, MN, USA) as control-Ig, both at 10 μg/mL; cyclosporin A, tacrolimus, sirolimus (all three LC Laboratories, Woburn, MA, USA), everolimus or MMF (both Selleckchem, Houston, TX, USA), used at 10 μM. DMSO (Carl Roth, Karlsruhe, Germany) was used as solvent control and PHA (phytohaemagglutinin, Roche, Mannheim, Germany, 5 μg/mL) as positive control. EBV-derived VLPs and extracellular vesicles (EVs) from HEK293 cells without any EBV protein, used as negative control particles, were added to PBMC at concentrations of 10 μg/mL. IFN-γ spots were quantified by Immunospot S5 UV-Reader, 5.1 software (CTL, Shaker Heights, OH, USA). All experiments were normalized to 250.000 cells/well, values for CEF or allo-specific stimulation were set to 100% and values for inhibitor treatment were calculated as IFN-γ spots [%], respectively.

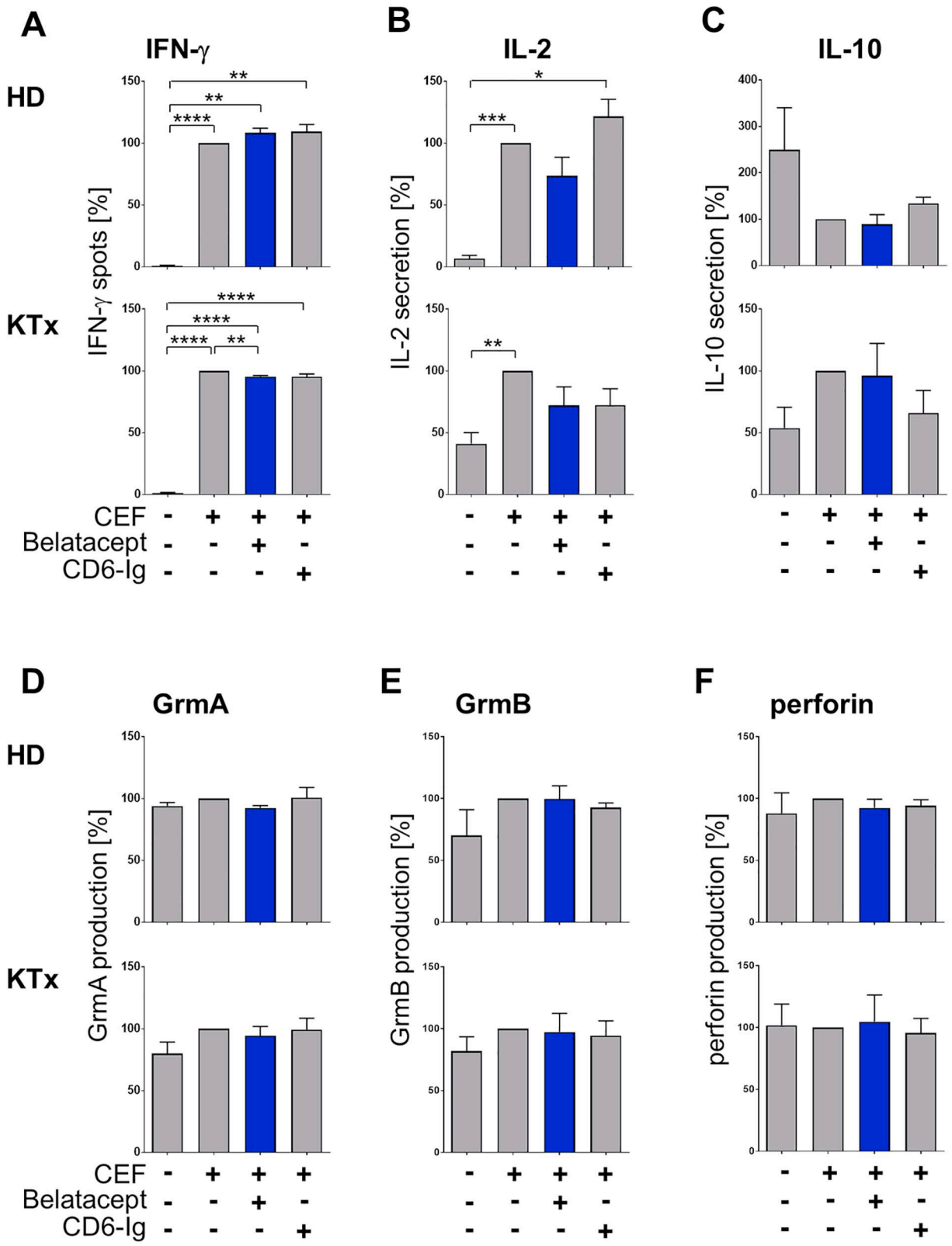

Fig. 1. Costimulation blockade by Belatacept preserves virus-specific IFN-γ T cell responses in KTx and has no impact on preformed molecules.
PBMC of healthy donors (HD, $n = 3$) or kidney transplanted patients (KTx, $n = 7–8$) were stimulated with CEF-peptides (500 ng/mL) for 18 h. Belatacept or CD6-Ig (control) was added at 10 μg/mL, IFN-γ producing cells were quantified by ELISpot (A), and supernatants were tested in parallel for secretion of IL-2 (B) and IL-10 (C), Grm A (D), GrmB (E) and perforin (F) by the Luminex-based multiplex technology. Results were normalized to CEF stimulation. Shown are the means ± SEM, for the statistics, ANOVA with Tukey's multiple comparison test was performed, asterisks indicate the p-values, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ and $^{****}p < .0001$.

2.3. Quantification of cytokines by multiplex technology

ELISpot supernatants were collected and analyzed for IL-2, IL-10, perforin, granzyme A/B, and soluble Fas ligand (sFasL) using human CD8$^+$ T cell kit (HCD8MAG-15 K, Merck/ Millipore, Darmstadt, Germany), and the Luminex-based multiplex technique according to the manufacturer's instructions. Standard curves and concentrations were calculated from > 50 beads per analyte and sample using Bio-Plex Manager 6.1 software (BioRad, Hercules, CA, USA).

2.4. Statistical analyses

D'Agostino Pearson omnibus normality test was used to assess data distribution. Statistical analyses were performed as indicated in figure legends with $p < .05$ considered significant. All statistical analyses were calculated with GraphPad Prism (Version 7, LaJolla, CA).

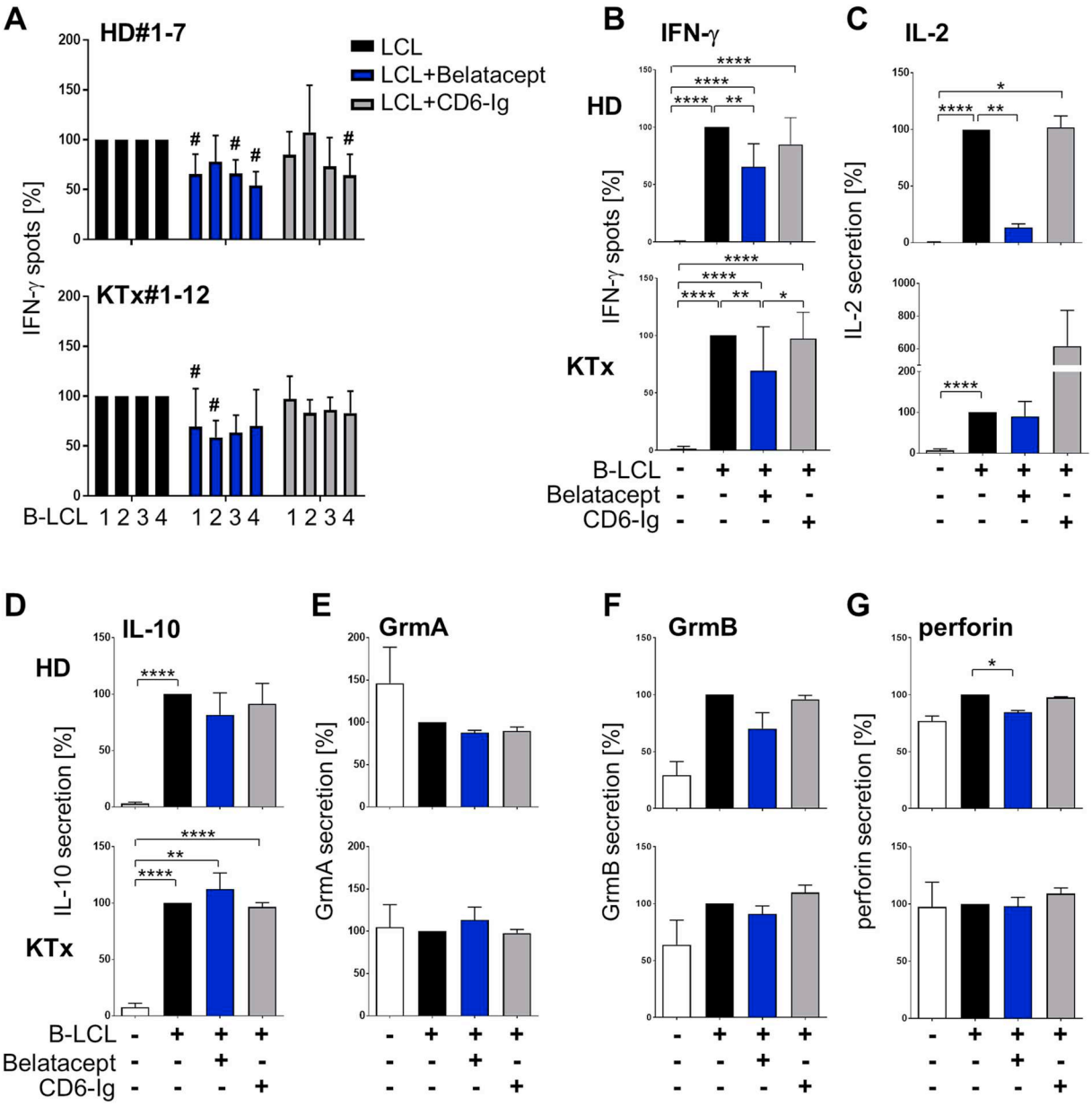

Fig. 2. Belatacept inhibits allo-specific IFN-γ and IL-2 responses but does not influence GrmA, B or perforin.
PBMC of healthy donors (HD, n = 3–7) or kidney transplanted patients (KTx, n = 7–12) were stimulated with four different allogeneic B cell lines (B-LCL) for 18 h. Belatacept or CD6-Ig was added at 10 μg/mL, IFN-γ production was measured by ELISpot and is shown for individual LCL stimulator cell lines for HD (top) and KTx (bottom), respectively (A). PBMC of HD or KTx were stimulated with one allogeneic B cell line (B-LCL) as in (A) and IFN-γ production was measured by ELISpot (B). Supernatants from (B) were tested in parallel for IL-2 (C) and IL-10 (D), granzyme A (E), granzyme B (F) and perforin (G) by Luminex-based multiplex technology. Results were normalized to allogeneic stimulation. Shown are the means ± SEM, for the statistics, ANOVA with Sidak's multiple comparison test was performed, hash indicates significant differences (# $P < .05$) compared to respective B-LCL alone, asterisks indicate the p-values, *$p < .05$, **p < .01, ***p < .001 and ****p < .0001.

3. Results

3.1. Belatacept preserves virus-specific memory T cell responses in healthy donors and KTx patients

To investigate the efficacy of costimulation blockade with Belatacept on T cell activation, IFN-γ ELISpots with PBMC of healthy donors (HD) or kidney transplanted (KTx) patients were performed. Additionally, the release of cytokines and cytotoxins was quantified in these ELISpot supernatants. PBMC were stimulated with a pool of CMV, EBV or influenza-derived (CEF) peptides to quantify the virus-specific memory $CD8^+$ T cell response (Table 2). Despite their immunosuppression, KTx patients did not show lower IFN-γ responses to CEF peptides compared to PBMC of HD. In PBMC of HD and KTx patients, costimulation blockade by Belatacept had no suppressive effect on IFN-γ induction by virus-specific $CD8^+$ memory T cells, and no effect was observed with the CD6-Ig control protein (Fig. 1A, and Fig. S1A). Functionality of Belatacept and the CD6-Ig fusion protein was demonstrated by staining of an EBV-transformed B cell line expressing both ligands (Fig. S1B). In addition, part of the IFN-γ $CD8^+$ T cell response in KTx recipients was shown to be CMV peptide-specific (Fig. S1C). Following expansion, CMV-specific $CD8^+$ T cells have down-regulated CD28, a hallmark of memory T cells, whereas naïve $CD8^+$ T cells still express CD28 along with CD27 (Fig. S1D). With respect to other Th1 cytokines, Belatacept was also unable to modulate IL-2 secretion by virus-specific memory T cells in HD and KTx patients (Fig. 1B). The Th2 cytokine IL-10 was constitutively secreted in HD and induced in KTx patients upon virus-specific stimulation and Belatacept

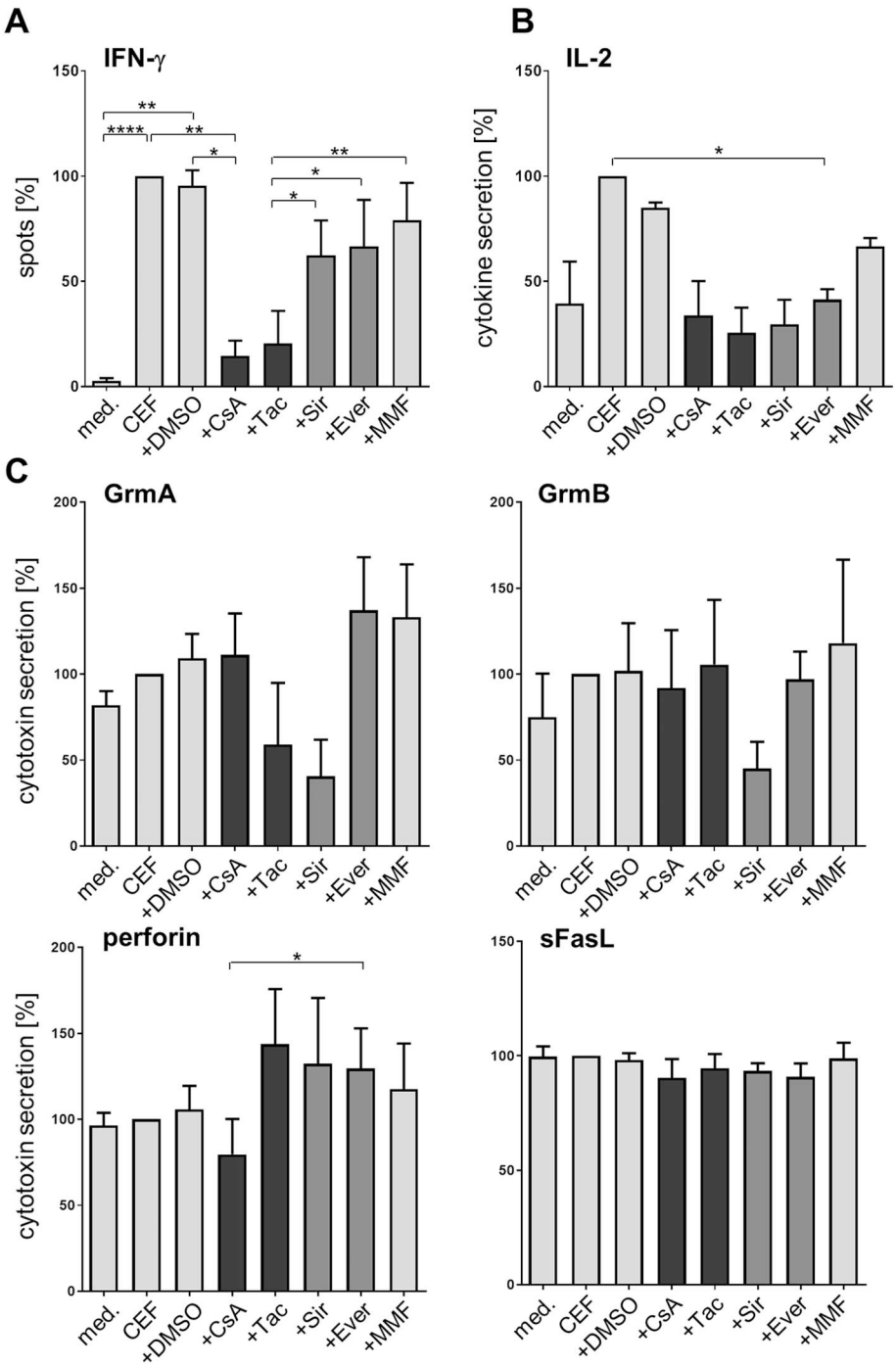

Fig. 3. Calcineurin inhibitors prevent virus-specific IFN-γ production while preformed molecules are unaltered.
PBMC of healthy donors were left untreated (medium), stimulated with CEF peptides alone (CEF; 500 ng/mL) or in combination with the solvent DMSO, with calcineurin inhibitors (CsA and Tac), mTOR inhibitors (sirolimus, Sir and everolimus, Ever) or MMF, each with 10 μM for 18 h. IFN-γ ELISpots ($n = 4$) were performed (A), and respective supernatants (n = 3) were tested for IL-2 (B) and preformed molecules like GrmA, B, perforin and sFasL (C). Results were normalized to CEF stimulation. Shown are mean ± SEM values, for the statistics, ANOVA with Tukey's multiple comparison test was performed, asterisks indicate the p-values, *p < .05, **p < .01 and ****p < .0001.

could also not suppress this IL-10 release (Fig. 1C, and Fig. S1A).

To examine whether CD28 costimulation blockade by Belatacept may have an effect on cytolytic effector functions of $CD8^+$ T cells, secretion of cytotoxins like granzyme (Grm) A, B and perforin was analyzed in ELISpot supernatants. In T cells, cytotoxins are constitutively expressed, stored in granules and released upon TCR-mediated activation in the course of degranulation and, hence, cytotoxicity is not transcriptionally regulated like cytokine, *i.e.* IFN-γ expression. Release of GrmA/B and perforin could not be enhanced significantly upon stimulation with virus-specific peptides and neither Belatacept, nor CD6-Ig was able to inhibit this cytotoxin release (Fig. 1D-F, and Fig. S1A).

3.2. De novo allo-specific T cell responses are inhibited by costimulation blockade while preformed cytotoxic molecules are not altered

In parallel to the virus-specific stimulations described in Fig. 1, the effectiveness of costimulation blockade was also analyzed with respect to *de novo* allo-specific $CD4^+$ and $CD8^+$ T cell responses. PBMC of either HD or KTx patients were stimulated with allogeneic EBV-transformed B-cell lines (B-LCL) from four different donors (supplemental Table S1). Costimulation blockade by Belatacept showed suppressive effects on the *de novo* allo-specific IFN-γ production by PBMC of HD and KTx patients compared to the addition of CD6-Ig control protein, reaching significance for three and two of the four B-LCL target cells, respectively (Fig. 2A). In parallel to the *de novo* IFN-γ production in response to one representative B-LCL (Fig. 2B), IL-2 secretion was also suppressed by Belatacept in this allogeneic setting reaching significance only in the HD but not the KTx group (Fig. 2C). In contrast to IFN-γ and IL-2, IL-10 was also induced upon allogeneic stimulation but neither suppressed by Belatacept nor by CD6-Ig (Fig. 2D). With respect to the cytotoxins GrmA and B, a constitutive release into supernatants was observed in the absence of allogeneic B-LCL target cells, which was not reduced by Belatacept or CD6-Ig (Figs. 2E-G). A small suppressive effect by Belatacept was detected for perforin secretion of HD back to baseline levels without stimulation ($p = .0246$, Fig. 2G). In contrast to the cytokines shown in Figs. 1A-C and 2A-D, the concentrations of GrmA/B and perforin did not differ between virus- and allo-specific stimulations, demonstrating that these cytotoxins may be produced constitutively, independently from TCR stimulation and, hence, resistant to costimulation blockade (Fig. S2).

3.3. CNI but not mTORI inhibit virus-specific cytokine secretion

As shown in suppl. Fig. S2, long-term immunosuppression in kidney transplanted patients seems to influence allo-specific production of IFN-γ, IL-2 and IL-10. This tendency, though weaker, was also observed for virus-specific IL-2 production. To investigate the efficacy of CNI and mTORI on T cell responses *in vitro*, PBMC of healthy donors were stimulated with CEF peptides in the presence or absence of the immunosuppressive drugs, *i.e.* CsA, Tac, sirolimus (Sir), everolimus (Ever) and MMF (mycophenolate-mofetil). While both calcineurin inhibitors could significantly suppress virus-peptide-specific IFN-γ production, reaching significance for CsA ($p = .0086$), mTOR inhibitors and MMF had no significant suppressive effect (Fig. 3A). Secretion of IL-2 was suppressed by both CNI and mTOR inhibitors with significance for everolimus ($p = .0306$) (Fig. 3B), MMF had no significant effect. For IL-10 secretion, an induction, though not significant, could be seen in the presence of CNI whereas all other immunosuppressive drugs showed no effect (Fig. S3A). Analogously to Belatacept, CNI did not influence the release of cytotoxins and/or soluble Fas ligand (sFasL), a cytotoxic death receptor ligand (Fig. 3C), again indicating that these cytolytic proteins were not transcriptionally regulated but rather stored in preformed granules and released upon activation.

3.4. Cytokine secretion of KTx patients is comparable to healthy donors following unspecific stimulation

To test the general capacity for cytokine secretion of PBMC of kidney transplanted patients *versus* HD, PBMC were stimulated with PHA. There were no significant differences regarding production of transcriptionally regulated cytokines like IFN-γ, IL-2 and IL-10 (Figs. 4A, B). The release of the cytotoxins GrmA, B, perforin and sFasL from intracellular granules, which is independent from transcriptional regulation, did also not differ between healthy donors and KTx patients (Fig. 4C). These observations support the rationale that T cell responses vary depending on the respective pathway for stimulation (virus-, allo-specific or *via* PHA) and that the strength of TCR and other receptor signals determines the functional efficacy of immunosuppression in kidney recipients as well as healthy individuals.

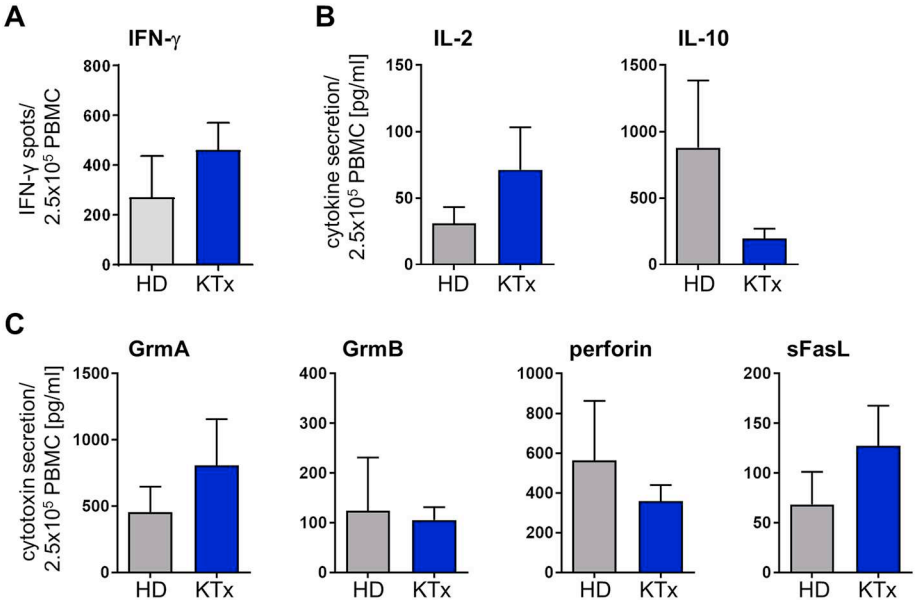

Fig. 4. Response capability of PBMC of healthy donors and kidney transplanted patients does not differ significantly.
PBMC of healthy donors (HD, n = 3) or KTx patients (KTx, $n = 7–8$) were stimulated with PHA (5 μg/mL) for 18 h. IFN-γ was detected *via* ELISpot (A); IL-2, IL-10 (B), GrmA, B, perforin and sFasL (C) were measured in the respective supernatants *via* Luminex based multiplex analysis. Results were normalized to 2.5×10^5 PBMC per well. Means ± SEM are shown, unpaired *t*-test with Welch's correction was performed for the statistics.

3.5. VLPs induce weak cytokine responses in kidney recipients and healthy donors

Epstein-Barr virus like particles (EBV-VLPs) represent an important EBV-specific vaccine strategy for immunosuppressed patients. In mice, these particles generated an EBV-specific immune response as shown by the groups of Hammerschmidt and Zeidler [12]. In order to address the immunogenicity of EBV-VLPs in humans, IFN-γ ELISpots were performed using PBMC of KTx patients or HD either in the presence of EBV-VLPs or exosome vesicles (EVs) as negative control. EBV-VLPs but not control EVs could induce weak IFN-γ production in PBMC of both groups, reaching significance only for KTx patients (mean 3,6 fold, $p = .0236$) due to the higher variability of the response in HD (mean 14,5 fold, $p > .05$; Fig. 5A). As additional read-out, cytokine- and cytotoxin-production was quantified also in these ELISpot supernatants (Figs. 5B-G). While IL-2 secretion upon EBV-VLP stimulation was only weakly induced in HD but not in KTx patients, significantly higher production of IL-10 was detected in supernatants of KTx patients ($p = .001$, Figs. 5B, C). The cytotoxins GrmA, B, perforin and sFasL were again constitutively released without increase upon exposure to EBV-VLPs or EVs without EBV antigens (Figs. 5D-G). The observation that EBV-VLPs can elicit a weak but detectable IFN-γ T cell response in both kidney recipients and healthy donors supports this strategy for development of a novel vaccine.

4. Discussion

In solid organ transplantation, particularly in kidney transplantation, there is a strong medical need for an improvement of immunosuppression by reducing side effects like kidney toxicity while simultaneously maintaining immune competence to prevent from viral infections. Here, we investigated virus- *versus* allo-specific T cell responses in PBMC of kidney transplanted patients and healthy donors, respectively, and compared the suppressive capacity of costimulation blockade *via* Belatacept, *i.e.* CTLA-4-Ig, with CNI and mTOR inhibitors. PBMC of KTx patients and HD were selected according to their HLA alleles with respect to presentation of virus-specific peptides (Table 2). Despite the immunosuppression, both virus- and allo-specific IFN-γ T cell responses were detectable at similar levels in KTx patients compared to healthy donors (Figs. 1, 2). While Belatacept could not suppress virus-specific IFN-γ production by T cells of KTx or healthy donors, this memory T cell response was still sensitive to treatment with CNI but not mTORI *in vitro* (Fig. 3A). However, *de novo* allo-specific induction of IFN-γ by T cells was only significantly reduced in KTx patients without reaching significance in HD. This difference argues for a slightly higher dependence of naïve allo-specific T cells from CD28-mediated costimulation than of virus-specific memory T cells. In our HLA/peptide specific setting, we could not confirm the findings by Xu et al. who found higher allo-specific production of IL-2 and IL-10 compared to the virus-specific response [13]. Moreover, they could also demonstrate enhanced proliferation and increased cytokine production

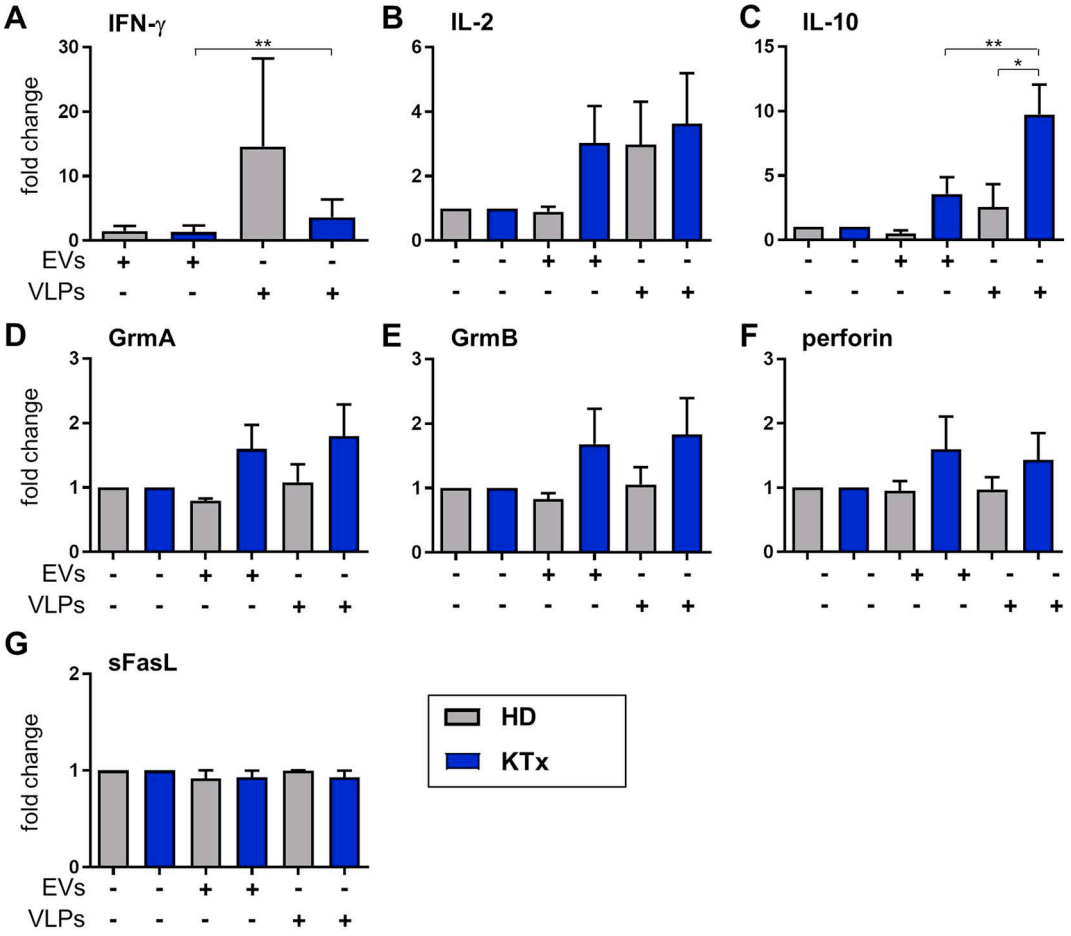

Fig. 5. EBV-VLPs induce a weak IFN-γ response in HD reaching statistical significance in kidney transplant patients.
PBMC of healthy donors (HD, n = 3–7) or kidney transplanted patients (KTx, $n = 8–12$) were either stimulated in the presence of EVs or EBV-VLPs (each with 10 μg/mL) for 18 h. IFN-γ was detected *via* ELISpot (A); IL-2 (B), IL-10 (C), granzyme A (D), granzyme B (E), perforin (F) and sFasL (G) were measured in the ELISpot supernatants *via* Luminex based multiplex analysis. Results were normalized, means ± SEM are shown, paired and unpaired t-test with Welch's correction, respectively, were performed for the statistics, asterisks indicate the p-values, *$p < .05$ and **$p < .01$.

(IFN-γ, TNF-α) in healthy donors upon allo-specific compared to virus-specific stimulation [13]. It is generally accepted that higher allo-specific T cell responses are likely to result from higher precursor frequencies of allo-reactive compared to virus-specific T cells. This holds also true for KTx patients whose capacity to elicit T cell responses is rather impaired as a result of their immunosuppression. In line with our observation of a differential impact of Belatacept on allo- rather than virus-specific T cells, the group of Baan [14] demonstrated inhibition of allo-specific IFN-γ production by $CD4^+$ and $CD8^+$ effector memory T cells by Belatacept in a dose-independent manner. In contrast, Xu et al. showed a dose-dependent suppression of allo-specific IFN-γ and TNF-α production, reaching significance only at an exceptionally high concentration of 100 μg/mL. All studies share the observation of Belatacept being unable to completely suppress allo-specific cytokine production. In our setting we used 10 μg/mL of Belatacept and at this concentration Xu et al. did not observe a significant downregulation of cytokine-producing cells [13]. As we saw a suppressive effect on the allo-specific IL-2-production at this concentration, as well as based on the rare patient material, we decided, to perform the IFN-γ ELISpots with this concentration only. It seems that IL-2 is most dependent on CD28-costimulation in comparison to the other cytokines analyzed [15]. Unfortunately, the group of Xu et al. did not show their results for IL-2 single producing T cells.

Compared to the dose-dependent suppressive capacity of allo-specific reactions, Belatacept weakly diminished the virus-specific IFN-γ response directed against a mixture of CMV, EBV and influenza-peptides. For CMV, this could be confirmed by Xu et al., also for the highest used Belatacept concentration. The discrepancy between the suppressive capacities of virus- and allo-specific immune reactions by Belatacept is most likely due to the *de novo* induction of an allo-response in our setting while the virus-specific immune reaction is based on the activation of memory effector $CD8^+CD28^-$ T cells. It is known since many years that antigen experienced T cells are independent of CD28 costimulation compared to naïve T cells [16,17]. During differentiation [18] and aging [15], CD28 is downregulated on $CD4^+$ and $CD8^+$ T cells, while both $CD4^+$ [19] and $CD8^+$ [18] T cells acquire expression of CD57, a marker for senescence, with progressing differentiation. Additionally people aged over 80 years have 50–60% $CD8^+$ $CD28^-$ T cells within their $CD8^+$ T cell compartment [20]. Especially for common chronic virus infections like CMV and EBV it is known that $CD8^+$ $CD28^-$ T cells are enriched in these infected patients and as Belatacept acts highly specific on the CD28–B7.1/2 axis, such cells should consequently not be inhibited by this drug. Still, it can be beneficial for Belatacept treated patients because of their inherent capacity to react to viruses and other memory effector T-cell based immune reactions should be comparable to HD. Besides, it could be a risk factor since individuals with high frequencies of $CD4^+$ or $CD8^+$ $CD28^-$ T cells pre transplant may be more prone to experience episodes of graft rejection under Belatacept treatment [14,19,21].

We could show that Belatacept did not influence the release of cytotoxins like granzyme A and B after virus- or allo-specific stimulation (Figs. 1 and 2), neither in HD nor in kidney transplanted patients. These molecules are preformed and stored in vesicles to be released very fast upon stimulation [22], while interference of CD28 costimulation is very unlikely to have an effect on the release itself. On the contrary, allo-specific perforin production is significantly diminished to baseline level by Belatacept in HD. Interestingly, perforin upregulation is not observed before day 6 after activation [23], and during short-term activation (as performed in our assays) even a downregulation of perforin mRNA has been described [24]. On the contrary, upon secondary stimulation of murine $CD8^+$ T cells, TCR restimulation induces perforin mRNA and protein rapidly after 6 h [24]. The general weak induction of perforin in our system is probably owing to the very low frequency of virus-specific memory $CD8^+$ T cells respectively naïve allo-specific $CD8^+$ T cells in humans as compared to the murine system, especially when a TCR transgenic mouse strain is used. Unfortunately, the direct effect of CD28 costimulation on production of cytotoxins like perforin and granzyme A/B is not investigated in the literature. Since perforin expression is strongly dependent on IL-2 [24], CD28 blockade may have a decreasing effect, probably seen after long-term stimulation for several days as IL-2 production itself is dependent on CD28 costimulation.

As we saw an effect of immunosuppression in case of KTx patients, we investigated the influence of commonly used drugs like CNI, mTOR inhibitors and MMF on virus-specific stimulated PBMC of HD. Only the two CNI were able to significantly decrease virus-specific IFN-γ production whereas IL-2 and IL-10 production could not be blocked by all tested inhibitors (Fig. 3 A/B). In general, in our hands CNI seem to be more potent in suppressing cytokine responses than other immunosuppressive drugs. Also, in previous own experiments, we demonstrated PMA/ionomycin- induced IFN-γ, TNF-α, IL-13 and IL-22 production by PBMC and isolated NK cells can only be successfully inhibited by cyclosporin A and tacrolimus [4]. As seen for Belatacept, also CNI and mTORI were not able to suppress cytotoxins like perforin and granzyme A/B (Fig. 3 C), probably again because these molecules are not transcriptionally regulated, but preformed in vesicles and only secreted upon stimulation. In support of our findings, other publications also described decreasing effects of CNI on NK cell degranulation [25] and no or even a slightly increasing effect [26]. Hodge and colleagues found physiologically relevant concentrations of CNI to decrease granzyme B production of CD3/CD28-stimulated $CD4^+$ and $CD8^+$ T cells partially but nevertheless significantly and GrmB inhibition by CNI was incomplete [27].

We could show in earlier experiments [4], that PMA/ionomycin-induced IFN-γ production of PBMC of KTx patients and HD are comparable despite the underlying immunosuppression. This could also be confirmed for stimulation with Phytohaemagglutinin (PHA), a T cell mitogen unspecifically activating T cells and, more interesting, for virus- and allo-specific stimulation *via* CEF-peptides and an allogeneic B cell line, respectively (Fig. 4, suppl. Fig. 1).

In transplanted, immunocompromized patients, Epstein-Barr Virus (EBV) infection or reactivation is an immense risk as immunosuppressed EBV-specific $CD4^+$ and $CD8^+$ T cells cannot control proliferation of EBV-infected B cells [12]. Therefore, we tested the capability of EBV-VLPs to induce a $CD8^+$ T cell driven *de novo* response. In both, HD and KTx patients, EBV-VLPs induced a weak IFN-γ production reaching statistical significance in KTx (Fig. 5A), no relevant IL-2 production and at least in KTx patients a weak IL-10 production (Fig. 5B). Cytotoxins like perforin and granzyme A/B could not be induced by EBV-VLPs (Fig. 5C). By using exactly the same VLPs, Ruiss and colleagues also observed a weak IFN-γ response either by stimulating a $CD8^+$ EBV-specific T cell clone with EBV-VLP preincubated PBMC respectively proliferation of $CD4^+$ T cells of EBV seropositive volunteers after three rounds of restimulation with EBV-VLP pulsed irradiated autologous PBMC [12]. The group of Ogembo constructed and investigated the vaccinating capacity of different EBV-VLPs containing diverse EBV proteins in mice [28]. In their system, a VLP comprising gH/gL and EBNA1 was the most potent IFN-γ inducing vaccine. The key difference between our study and the cited publications is measuring of the *de novo* response without any preincubation of cells with VLPs *versus* determination of a memory immune response after several rounds of immunization. Altogether our results demonstrate EBV-VLPs to be capable of inducing an EBV-specific T cell response, most importantly also in immunosuppressed patients yet several rounds of immunization may be required for an efficient vaccination.

5. Conclusions

In this study, we could demonstrate successful suppression of allo- but not virus-specific IL-2 responses by Belatacept. This is a major advantage of drugs like Belatacept functioning specifically on T cell activation. Moreover, due to its narrow activity spectrum, Belatacept nearly has no side effects compared to immunosuppressant drugs like

CNI or mTORI that act on commonly used cellular signaling pathways and contribute to renal toxicity. The capability to react quite normal to virus infections is very important for transplanted patients while simultaneously the allo-response against the graft needs to be controlled. In line with this fact, the possibility to immunize immunosuppressed patients against common viruses like EBV is very important and EBV-VLPs would represent a promising novel vaccination strategy for such patients. However, the EBV-VLPs tested in this work showed a weak potency to induce a *de novo* $CD8^+$ response, their behavior after several restimulations would be promising.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2020.101291.

Disclosure

The authors declare no conflict of interest.

Acknowledgments

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