Cross-Reactive Donor-Specific CD8⁺ Tregs Efficiently Prevent Transplant Rejection

Graphical Abstract

Highlights
- Human and rat CD8⁺ Tregs recognize long peptides derived from donor MHC class II molecules
- A cross-reactive CD8⁺ Treg population recognizes different MHC class II-derived peptides
- In vivo tolerogenic peptide vaccination induces CD8⁺ Tregs and transplantation tolerance

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In Brief
Picarda et al. describe MHC class II-derived peptides recognized by cross-reactive CD8⁺ Tregs instrumental for tolerance induction in transplantation between an incompatible donor and recipient.

Data Resources
6NF7

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Cross-Reactive Donor-Specific CD8⁺ Tregs Efficiently Prevent Transplant Rejection

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SUMMARY

To reduce the use of non-specific immunosuppressive drugs detrimental to transplant patient health, therapies in development aim to achieve antigen-specific tolerance by promoting antigen-specific regulatory T cells (Tregs). However, identification of the natural antigens recognized by Tregs and the contribution of their dominance in transplantation has been challenging. We identify epitopes derived from distinct major histocompatibility complex (MHC) class II molecules, sharing a 7-amino acid consensus sequence positioned in a central mobile section in complex with MHC class I, recognized by cross-reactive CD8⁺ Tregs, enriched in the graft. Antigen-specific CD8⁺ Tregs can be induced in vivo with a 16-amino acid-long peptide to trigger transplant tolerance. Peptides derived from human HLA class II molecules, harboring the rat consensus sequence, also activate and expand human CD8⁺ Tregs, suggesting its potential in human transplantation. Altogether, this work should facilitate the development of therapies with peptide epitopes for transplantation and improve our understanding of CD8⁺ Treg recognition.

INTRODUCTION

The therapeutic tolerogenic potential of regulatory T cells (Tregs) has been highlighted by recent studies in numerous fields, including transplantation, and several clinical trials using polyclonal Tregs have started (Fuchs et al., 2018; Tang and Vincenti, 2017). However, it has been shown that antigen-specific Tregs (both CD4⁺ and CD8⁺) have greater potential than unspecific polyclonal Tregs to control graft rejection and even induce tolerance (Filippe et al., 2019; Masteller et al., 2006; Picarda et al., 2011, 2014; Sagoo et al., 2011; Bézie et al., 2019). Thus, antigen

therapy appears to be a promising donor-specific therapeutic for the prevention of transplant rejection, which could diminish or even replace the non-specific immunosuppressive drugs that are so deleterious for the patient health in the long term (Marcén, 2009; Meier-Kriesche et al., 2006; Picarda et al., 2011, 2014). However, principles for the design and choice of donor-derived peptides to induce immunoregulation are unknown, and donor-derived peptides recognized by CD4⁺ and CD8⁺ Tregs have not been identified in human transplantation. So far, donor blood transfusion protocols in animal models have resulted in highly efficient donor-specific tolerance induction in a CD8⁻ or CD4⁻ dependent manner (Douillard et al., 1999; Liu et al., 2004; Ueta et al., 2018; Vignes et al., 2000), but they are not clinically applicable and could trigger global alloresponses. Using libraries of donor-derived peptides, we previously identified in a rat model of tolerance the sequence of a dominant peptide derived from a donor major histocompatibility complex (MHC) class II (RT1.D³) molecule and presented by a MHC class I (RT1.A⁰) molecule that specifically triggered activation of CD8⁺ Tregs (Picarda et al., 2014). Despite being less studied than CD4⁺ Tregs (Bézie et al., 2018a), CD8⁺ Tregs can efficiently inhibit transplant rejection in rat models of cardiac transplantation (Bézie et al., 2015a; Guillonneau et al., 2007; Picarda et al., 2017) and in immunodeficient (SCID)-IL2rg⁻/⁻ (NSG) mouse models of human skin transplantation and xenogeneic graft versus host disease (GVHD) (Bézie et al., 2018b). We showed that immune therapy using this peptide alone efficiently inhibited fully incompatible cardiac allograft rejection in rat through direct induction of T cell receptor (TCR) Vβ11 skewed CD8⁺ Tregs (Picarda et al., 2014). Recently, the structure of a human-induced CD4⁺ Treg TCR was solved in complex with a proinsulin-derived peptide presented by a MHC class II molecule and demonstrated a 180° polarity reversal, challenging our understanding of TCR recognition (Beringer et al., 2015).

In this study, we identified and characterized MHC class II-derived peptides sharing a consensus motif in rat and human, forming a unique structural complex with MHC class I, and
activating cross-reactive CD8+ Treg function in vitro and in vivo upon vaccination, which induced graft tolerance. These findings improve our understanding of antigen presentation and specificity to Tregs and could facilitate the development of therapies with peptide epitopes for transplantation.

RESULTS

A Self-MHC Class I Molecule Accommodates Long Peptide from a Donor MHC Class II Molecule with a Mobile Central Section Necessary to Activate CD8+ Treg Function

We previously described a library of 16 amino acid (aa)-long overlapping peptides generated from polymorphic domains of the donor MHC molecules of LEW.1W rats (Picarda et al., 2014). We showed that peptide 51 (further called Du51, sequence NREEYARFDSDVGEYR) and peptide 31 (further called Bu31, sequence YLRYDSDVGEYR) derived from the β1 domain of donor MHC class II RT1.Du and RT1.Bu, respectively, induced highly significant upregulation of CD25 expression at the cell surface of CD8+CD45RClow Tregs isolated from a tolerant grafted recipient (treated at day 0 with CD40Ig, a chimeric molecule blocking the CD40-CD40L pathway) after 6 days of culture in the presence of matured syngeneic plasmacytoid dendritic cells (pDCs) (Picarda et al., 2014) (Figure S1A). Both peptides shared an 8 aa motif (DSDVGEYR) that is potentially recognized by CD8+ Tregs.

To determine the sequence of the natural donor Bu31 peptide recognized by antigen-specific CD8+ Tregs, we designed a library of 9- to 15-mer degenerated peptides derived from 16-mer Bu31 and 32 peptides (labeled 31-1 to 31-15) (Figure 1A), taking into account the preferential presence of arginine (R) at the C terminus for binding to the MHC class I RT1.Aa molecule (Powis et al., 1996; Stevens et al., 1998a, 1998b; Thorpe et al., 1995). The library was tested in the same in vitro assay described earlier. Although none of the 9-mer derivative peptides 31-1 to 31-8 activated CD8+ Tregs, four longer derivatives—31-10, 31-13, 31-14, and 31-15—induced strong CD25 upregulation to a similar level than did Bu31 peptide. Interestingly, they all shared the 7 aa motif SDVGEYR, also present in the previously described Du51 peptide (sequence NREEYARFDSDVGEYR) and the shortest Bu31-10 peptide, with a thermal melting (denaturation) temperature of ~62°C, which was ~12°C higher than either one of the two 16-mer peptides in complex with RT1.Aa (Table S1). Interestingly, the 12-mer...
Bu31-10 peptide shares the same P2-Leu and P0-Arg residues as 16-mer Bu31 and Du51 peptides, respectively. Next, we solved the structure of the RT1.A\(^\alpha\) molecule in complex with the most stable Bu31-10 peptide at a resolution of 2.9 Å (PDB: 6NF7) (Table S2). The RT1.A\(^\alpha\)-Bu31-10 complex crystallized in the P2\(_1\) space group, with five peptide-MHC (pMHC) complexes in the asymmetric unit. Each of the five pMHC molecules showed an overall similar antigen-binding cleft conformation (root-mean-square deviation [RMSD] of 0.3 Å). The peptide exhibited a highly mobile conformation (Figure 1C) in the five complexes (RMSD of 0.5–0.9 Å), with weak electron density for the central part of the peptide that was not visible in the electron density (P5-DSDV-P8) (Figure S1B). Similar to previously solved structures of peptide-RT1.A\(^\alpha\) complexes, the RT1.A\(^\alpha\)-Bu31-10 complex showed a preferred small hydrophobic residue at P2 (Leu/Pro), a larger half-buried P3 residue (Arg/Phen), and a P0-Arg (Rudolph et al., 2002; Speir et al., 2001). The two anchor residues at P2 and P3 are shared with the less stable 16-mer Du51 and Bu31 peptides. The P0-Arg of Bu31-10 peptide forms a salt bridge with Asp116, Glu97, and Asp77 of the F pocket within the antigen-binding cleft (Figure 1D). Meanwhile, Bu31 peptide has a P0-Glu that is unfavorable in the negatively charged F pocket of RT1.A\(^\alpha\), which may explain the decreased stability of Bu31 peptide compared with Bu31-10 peptide (Table S1). The P2-Leu of Bu31-10 peptide sits above Tyr7 and Met45 of RT1.A\(^\alpha\) (Figure 1E); a larger residue such as the P2-Arg of Du51 peptide might lead to steric clashes within the B pocket of the RT1.A\(^\alpha\), which would be unfavorable. This might explain the lower stability of Du51 peptide compared with Bu31-10 peptide. Finally, the P3-Arg of Bu31-10 peptide, which is also shared with Bu31 peptide, formed a salt bridge with Glu97 and a hydrogen bond with Tyr152, further stabilizing the peptide within the RT1.A\(^\alpha\) molecule (Figure 1F). Collectively, our data show that the higher stability of Bu31-10 peptide results from favored anchor residues at positions P2, P3, and P0 that were partially missing within Bu31 or Du51 peptides. In addition, the structure of Bu31-10 peptide in complex with RT1.A\(^\alpha\) reveals a mobile central section of the peptide that might be stabilized upon TCR ligation and thus confirms the importance of the consensus sequence SDVGEYR present in each peptide.

A Cross-Reactive CD8\(^+\) Treg Population Recognizes Peptides Derived from Different MHC Molecules

To further understand the role of this consensus sequence in the mobile section, we generated MHC class I tetramers for RT1.A\(^\alpha\)/Bu31-10 (tet Bu31-10) and RT1.A\(^\alpha\)/Du51 (tet Du51) and tested specific binding of CD8\(^+\)CD45RClow Tregs from spleen, graft, and blood of cardiac graft recipients who had been tolerant for >120 days. Both PE- and APC-conjugated tet Bu31-10 or tet Du51 were used to discriminate a true signal from noise as described previously (Picarda et al., 2014). Non-specific binding to a BV421-labeled RT1.A\(^\alpha\)/MTF-E (Tet MTF-E) control tetramer was eliminated, together with dead cells (Figure 2A). Around 1.38% of CD8\(^+\)CD45RClow Tregs in the graft, 0.67% in the spleen, and 0.25% in the blood were specific for Bu31-10 peptide (Figures 2A, left panels, and 2B). This percentage is 3- to 5-fold lower than the one of dominant Du51-specific cells (Figures 2A, middle panels, and 2B) (Picarda et al., 2014). Analysis of potential Treg cross-reactivity showed that all Bu31-10-specific Tregs also cross-recognized peptide Du51 but a fraction of Du51-specific Tregs did not bind to tet Bu31-10 (Figures 2A, right panels, and 2B). Cross-reactive Tregs were mostly present in the graft, followed by the spleen and to a lesser extent the blood. Moreover, we showed that 20% to 40% of Du51 and Bu31-10 antigen-specific Tregs express FoxP3 (Figure 2C).

Given the non-canonical length of the peptide and the presence of the consensus sequence recognized by both Du51 and Bu31-10 antigen-specific Tregs, we next performed alanine scanning mutagenesis of the Du51 dominant peptide to determine the contribution of each residue to CD8\(^+\) Treg recognition and activation (Figure 2D). All alanine analogs showed a substantial reduction in Treg activation compared with wild-type peptide. Substitution of P16-Arg (R16A) resulted in the most significant loss of activation, which might result from its critical role in peptide stability. Substitutions at positions P3-Glu, P4-Glu, and P7-Arg and within the DSDVG peptide motif at P10-Ser and P12-Val also significantly reduced Treg activation, possibly because of peptide instability and defective TCR ligation as suggested by Bu31-10 structural analysis.

Altogether, these results demonstrated that a fraction of CD8\(^+\)CD45RClow Tregs harbored a flexible TCR capable of recognizing at least two distinct peptides but with a consensus sequence probably key to the recognition.

Stimulation with Bu31 Peptide Leads to Efficient CD8\(^+\)CD45RClow Treg Activation and Suppressive Function

Given the low frequency of Bu31-specific cells within the total CD8\(^+\)CD45RClow Treg population, we assessed the capacity of Bu31 peptide to stimulate and increase Treg function after 6 days of incubation with syngeneic pDCs (Figure 2E). Bu31-stimulated CD8\(^+\) Tregs upregulated their secretion of interferon gamma (IFN\(_g\)), an important cytokine for CD8\(^+\) Treg function (Guillonneau et al., 2007), as measured in the culture supernatant (Figure 2E) and their expression of CD25 and CD71 as measured by flow cytometry (Figures 2F and S1A). We also observed a trend for an increase in CD28 and MHC class II expression, but no change in Foxp3 level. After 6 days of Bu31 or control peptide stimulation through the indirect pathway of recognition, CD8\(^+\)CD45RClow Tregs were recovered and tested for their suppressive capacity in a secondary coculture of effector CD4\(^+\)CD25\(^-\) T cells stimulated by the direct allorrecognition pathway with donor pDCs as previously described (Picarda et al., 2014). In this assay, suppression depends on the efficacy of Treg pre-stimulation with peptide and relies on bystander suppression through secretion of inhibitory factors. Bu31-stimulated Tregs significantly suppressed effector T cells proliferation compared with Tregs cultured with a non-activating peptide (Figure 2G). Altogether, we showed Bu31 peptide efficiently activated CD8\(^+\)CD45RClow Tregs and potentiated their suppressive activity in vitro.

In Vivo Tolerogenic Bu31 Peptide Vaccination Induces Tolerance to a Fully Mismatched Cardiac Graft in Rat through CD8\(^+\)CD45RClow Tregs

To assess the potential of Bu31 peptide in in vivo generation of CD8\(^+\)CD45RClow Tregs and in allograft survival, animals were treated with the peptide as monotherapy at doses of 0.5 or
A

B

C

D

E

F

G

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1 mg/day for 28 days, starting day 7 before transplantation and constantly released intraperitoneally (i.p.) by mini-osmotic pumps (Figure 3A). Although a dose of 0.5 mg/day was not sufficient to delay cardiac graft rejection, a higher dose of 1 mg/day of peptide induced indefinite allograft survival in 80% of recipients (**p < 0.01 compared with 0.5 mg/day and no treatment) (Figure 3B). Long-survival graft induction was donor specific, because third-party Brown Norway (BN) grafts of a different haplotype (RT1b) that did not contain the conserved motif were quickly rejected. In addition, tolerance induced by peptide vaccination depended on CD8+ T cells, because recipients depleted of CD8+ cells using a depleting anti-CD8 monoclonal antibody (mAb) (OX8 clone) (Bézie et al., 2015b; Guillonneau et al., 2007) also rapidly rejected their graft. Histologic analysis of the cardiac graft at day 120 following transplantation showed no signs of chronic rejection (Figure 3C) according to a score previously established (Bézie et al., 2015a; Guillonneau et al., 2007; Picarda et al., 2017). Furthermore, anti-donor humoral responses were abrogated in long-term Bu31-treated recipients (Figure 3D). Finally, we analyzed the suppressive capacity of CD8+CD45RClow Tregs from long-surviving tolerant recipients in vitro in the presence of effector CD4+CD25- T cells and donor pDCs (Figure 3E) and in vivo upon adoptive transfer in newly grafted irradiated recipients (Figure 3F). Tregs from Bu31-treated recipients were significantly more suppressive than naive cells (Figure 3E), with an almost total inhibition of effector CD4+ T cell proliferation at 1:4 and 1:2 effector:suppressor ratios. In vivo, adoptive cell transfer of either total splenocytes or CD8+CD45RClow Tregs from long-term tolerant Bu31-treated rats significantly delayed allograft rejection in secondary transplanted recipients compared with naive splenocytes (Figure 3F).

Overall, these results demonstrated that short-term tolerogenic vaccination starting before transplantation efficiently induced tolerance, inhibited cellular and antibody-mediated rejection, and improved CD8+CD45RClow Treg suppressive function.

16 aa Human MHC Class II-Derived Peptides Bearing the Conserved SDVGE-X-R Motif Activate Human CD8+CD45RClow Tregs

To better understand the relevance of our findings in human transplantation, we designed four 16 aa peptides from four random human histocompatibility leucocyte antigen (HLA) class II alleles based on their alignment with sequences of the rat Du51 peptide (NREEYARFSDVGEYR) and the overlapping 12-mer Bu31-10 peptide (YLRYSDVGEYR), both bearing the SDVGEYR motif at the C-terminal end (Figure 4A). We individually tested these human peptides differing at position 2, 5, 6, 14, or 15 in a 5-day culture assay using CD8+CD45RClow Tregs and autologous pDCs from the same individuals in the presence of interleukin (IL)-2 and CpG in serum-free Texmacs medium (Figure 4A). CD25 and CD69 expression was upregulated on Tregs following incubation with Hpep1, Hpep2, and Hpep4 peptides and reached statistical significance for Hpep2 (Figure 4B). These three peptides share the conserved SDVGE-X-R 7 aa motif; Hpep3 has a valine (V) in place of the glutamic acid (E) at p14 of the peptide, probably affecting TCR recognition. To determine whether Tregs could be expanded using such HLA class II-derived peptide, we set up an expansion protocol using sorted CD8+ Tregs and APCs from the same individual with the Hpep2 peptide in the presence of IL-2, IL-15, and CpG and compared the results with those of a polyclonal stimulation (anti-CD3/CD28 mAbs) (Bézie et al., 2018b) (Figure 4C). Hpep2 stimulation resulted in 8-fold expansion of total CD8+ Tregs in 14 days, although actual expansion of the small Hpep2-specific Treg fraction present at day 0 may be much higher (Figure 4D). Importantly, both Hpep2- and anti-CD3/CD28 mAb-stimulated Tregs retained their capacity to suppress an allogeneic immune response after expansion, similar to fresh CD8+ Tregs (Figure 4E). Peptide-stimulated Tregs tend to be more suppressive than polyclonal Tregs, suggesting the potential benefit of expanding antigen-specific Tregs for therapy. We did not observe significant differences in the level of expression of Foxp3, GITR, IL-10, IFNγ, and IL-34 in Hpep2 or anti-CD3/28 expanded CD8+ Tregs (Figure S2).

Altogether, HLA class II-derived peptides bearing the consensus SDVGE-X-R 7 aa motif efficiently activated and expanded suppressive human CD8+CD45RClow Tregs.

DISCUSSION

Understanding antigen presentation, recognition, and subsequent Treg activation and expansion is crucial for the development of therapeutic strategies for the treatment of numerous diseases.
Figure 3. Tolerogenic Vaccination with Bu31 Peptide Induces Allograft Tolerance through Potentiation of Specific CD8+ Tregs’ Suppressive Activity

(A) Tolerogenic vaccination protocol with mini-osmotic pump implantation in the abdomen of cardiac allograft recipient from day 7 before transplantation to day 21 post transplantation.

(B) Recipients were either untreated (n = 9) or treated with short-term continuous peptide infusion by i.p. mini-osmotic pumps delivering either 0.5 mg/day (n = 6) or 1 mg/day (n = 5) of Bu31 peptide alone or combined with a depleting anti-CD8α mAb (OX8) (n = 2) or 1 mg/day in the BN/LEW.1A strain combination (n = 4). Log rank test, *p < 0.05, **p < 0.01.

(C) Representative H&E histology of two representative sections of cardiac graft from long-surviving Bu31-treated rats at day 120 post-transplantation and treatment. Arrows indicate the intimal wall of the vessels. Original magnification, ×100.

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diseases, such as transplant rejection and auto-immunity. We previously demonstrated that a 16-mer-long dominant peptide derived from a donor MHC class II molecule strongly activated CD8+ Tregs (Picarda et al., 2014), contrasting with literature describing a more common short length preference for MHC class I. In the present study, we made a similar observation with a peptide derived from a different MHC class II molecule (16 aa original sequence and not less than a derivative of 12 aa). Historically, studies of random peptide libraries and of mass spectrometry have been limited to short lengths of 8 to 10 aa and have allowed the development of powerful tools for prediction of potential antigenic epitopes but rarely exceeded 11 aa. However, about 10% of presented peptides have a length greater than 11 aa (Burrows et al., 2006), even up to 25 aa (Bell et al., 2009). Recent studies suggest that MHC class I can accommodate peptides much longer than previously established, inviting broadening studies and peptide binding algorithms up to 15/16 aa. This clear preference for long peptides from two distinct MHC molecules observed for CD8+ Tregs is intriguing, and it would be interesting to know whether this is a general feature of CD8+ Tregs. Rist and colleagues demonstrated that HLA polymorphism affects the length of peptide presentation and CD8+ T cell response (Rist et al., 2013). It is not clear yet whether our finding regarding the characteristic of structural presentation is restricted to RT1.Au MHC class I presentation, known to accommodate long peptides (Stevens et al., 1998b), or whether it applies to other haplotypes in rat and human, as suggested by our results using long peptides in the human setting. Binding of long peptides on MHC class I has been shown in the same rat strain for a minor antigen peptide of 13 aa called MTF-E, and the structure revealed two conformations of the peptide, which forms a large and flexible bulge out of the MHC cleft (Speir et al., 2001), suggesting a high level of peptide flexibility that can be observed for long peptides in complex with MHC class I molecules (Chan et al., 2018; Ebert et al., 2009; Hassan et al., 2015; Josephs et al., 2017). Peptide length, and perhaps flexibility, might be important for CD8+ Treg recognition, because no peptide variant shorter than 15 aa for the RT1.Du and 12 aa for the RT1.Bu chains could efficiently activate CD8+ Tregs. In addition, usage of different anchor residues for the three peptides analyzed seems greater than what has been commonly described for T cell epitopes (Powis et al., 1996; Stevens et al., 1998a; Thorpe et al., 1995). In a tumor model, two distinct polyclonal TCRs recognizing a HLA class I-restricted dominant tumor epitope adopted differing TCR recognition modes, suggesting that extensive flexibility at the TCR-pMHC class I interface engenders recognition (Chan et al., 2018). An interesting feature of the different peptides we studied was the presence of a common motif DSDVGEYR, allowing recognition by a common pool of cross-reactive Tregs. Tetramer staining revealed that Bu31-10-specific CD8+ Tregs also recognized the Du51 peptide, suggesting either flexibility of the TCR recognition or focused recognition on the shared peptide motif. We previously showed that Du51-specific Tregs’ TCR was biased toward the Vβ11 family, suggesting that this may also be the case for Bu31-specific Tregs (Guillonneau et al., 2007; Picarda et al., 2014). We confirmed that the two allopeptides involved in acute rejection in the same mismatched cardiac allograft model did not activate CD8+ Tregs and that effector T cells isolated from rejecting untreated animals did not recognized the tolerogenic peptides (Ballet et al., 2009; Picarda et al., 2014).

The use of a soluble peptide as a therapeutic to decrease transplant rejection and selectively induce tolerance through antigen-specific Tregs was both feasible and efficient in our model. Although these Tregs were capable of potent bystander suppression in vitro, they did not prevent third-party allograft rejection in vivo, possibly because of a lower Treg:Teff ratio in vivo, stronger alloresponse to the third-party antigens, and the role played by innate immunity and B cells in rejection. Future work is needed to assess the detailed mechanism of tolerance induction in vivo and better understand the potential of bystander suppression mediated by antigen-specific Tregs and the roles played by IFNγ, transforming growth factor β (TGF-β), and IL-34. Although others have shown pMHC multimers can efficiently inhibit autoimmune diseases (Masteller et al., 2003), administration of tolerogenic pMHC multimers induced Treg apoptosis and graft rejection in our model (data not shown), suggesting a high avidity of the TCR for these tolerogenic multimers resulting in strong activation-induced cell death and the requirement for low-antigen doses in the context of transplantation (Mailone et al., 2004; Oling et al., 2010). Several studies have demonstrated the immunomodulatory effect of synthetic peptides derived from conserved regions of MHC molecules on alloimmune responses (Zang and Murphy, 2005): in vitro by inhibition of cell-cycle progression (Boytim et al., 1998) or induction of apoptosis (Murphy et al., 1999) and in vivo by inhibiting activation and effector function of alloreactive T cells in a mouse model of donor-specific transfusion (Murphy et al., 2003). In addition, administration of a peptide derived from the HLA-B7 molecule named Allotrap and associated with cyclosporine prolonged skin allograft survival in mice (Buelow et al., 1995) and heart allograft survival in rats while attenuating arteriosclerosis (Murphy et al., 1997). In mice, intratracheal administration of a 15-mer derived from the hypervariable region of H2-Kb prolonged cardiac allograft survival.

(D) Immunoglobulin G (IgG), IgG1, IgG2a, or IgG2b alloantibody production in naïve (n = 3), untreated (n = 3), or Bu31-treated (n = 4) animals > 120 days after transplantation. Graphs represent MFI ± SEM. Two-way ANOVA test, Bonferroni post-test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(E) In vitro CD8+ Treg suppressive activity from naïve or Bu31-treated cardiac allograft recipients following cell sorting > 120 days after transplantation. naïve (n = 5) or Bu31-CD8+ (n = 3) Tregs, CFSE-labeled CD4+CD25+ effector T cells, and allogenic pDCs were cocultured for 6 days. Proliferation of CD4+CD25− T cells in the control condition with pDCs and without Tregs (− 80%) was given the value 100 in each experiment. On the left, the graph represent mean ± SEM of the relative proportion of dividing CD4+ T cells. Two-way ANOVA test, Bonferroni post-test, ****p < 0.0001. On the right, one representative staining at ratio 1:1 for effector:suppressor is shown.

(F) 120 after transplantation, 150 × 10^6 total splenocytes or 2.5 × 10^6 sorted CD8+ Tregs from the spleen of naïve or Bu31-treated cardiac allograft recipients were adoptively transferred into naïve irradiated LEW.1A recipients transplanted with LEW.1W donor heart. Results are expressed as the percentage of allograft survival as monitored by palpation. Log rank test, *p < 0.05, **p < 0.01.
or induced tolerance, in combination with a non-depleting anti-human CD4 antibody (Aramaki et al., 2003), and generated regulatory cells. Finally, according to a pilot study in humans, oral administration of low doses of peptides derived from donor MHC (HLA-DR2) molecules to patients with chronic dysfunction of renal allograft induced complete inhibition of indirect alloreactivity in vitro, but suppression mechanisms have not been elucidated (Womer et al., 2008). Du51 and Bu31 peptides identified in our model have >90% homology with human HLA-DP, HLA-DQ, and HLA-DR molecules. To emphasize the clinical scope and potential future therapeutic interest of our work, we studied the response of human CD8+ Tregs to these homologous peptides (Bezie et al., 2018b). One peptide in particular bearing the consensus SDVGE-X-R 7 aa motif efficiently activated and expanded CD8+ Tregs. Importantly, Tregs retained their suppressive potential and tolerogenic phenotype after 14 days of expansion, suggesting this peptide specifically expanded Tregs, not effector T cells.

Figure 4. Long MHC Class II Peptides Activate and Expand Human CD8+ Tregs
(A) 16 aa peptides were designed on 4 human MHC class II alleles and tested for CD8+ Treg activation in 5 days of culture with HLA-A2+ syngeneic pDCs in medium supplemented with CpG and IL-2. Peptide mismatches are highlighted in red.
(B) CD8+ Treg activation in response to each peptide was quantified based on CD25 and CD69 expression. Bars ± SEM represent the fold change of positive cells after peptide stimulation compared with the control condition with an irrelevant peptide. Wilcoxon test versus 1, *p < 0.05, n = 9 to 14 for each peptide. Representative histograms are shown on the right.
(C) Protocol of CD8+ Treg expansion by Hpep2 peptide. CD8 Tregs were stimulated at day 0 and day 7 by syngeneic HLA-A2+ APCs, Hpep2 peptide, IL-2, IL-15, and CpG. Cytokines were added twice a week.
(D) Total Treg fold expansion after 14 days of culture with Hpep2 or anti-CD3/anti-CD28 mAbs (versus day 0). Individual samples and mean ± SEM are shown.
(E) After 14 days of peptide or polyclonal stimulation, expanded Tregs were tested for suppressive activity on syngeneic CFSE-labeled CD4+CD25+ T cells stimulated with allogeneic APCs pooled from 3 healthy volunteers, compared with fresh Tregs. Bars ± SEM represent the relative proportion of dividing CD4+CD25- T cells. ***p < 0.001, n = 2 to 5. Representative histograms are shown on the right.

(Akiyama et al., 2002) or induced tolerance, in combination with a non-depleting anti-CD4 antibody (Aramaki et al., 2003), and generated regulatory cells. Finally, according to a pilot study in humans, oral administration of low doses of peptides derived from donor MHC (HLA-DR2) molecules to patients with chronic dysfunction of renal allograft induced complete inhibition of indirect alloreactivity in vitro, but suppression mechanisms have not been elucidated (Womer et al., 2008). Du51 and Bu31 peptides identified in our model have >90% homology with human HLA-DP, HLA-DQ, and HLA-DR molecules. To emphasize the clinical scope and potential future therapeutic interest of our work, we studied the response of human CD8+ Tregs to these homologous peptides (Bezie et al., 2018b). One peptide in particular bearing the consensus SDVGE-X-R 7 aa motif efficiently activated and expanded CD8+ Tregs. Importantly, Tregs retained their suppressive potential and tolerogenic phenotype after 14 days of expansion, suggesting this peptide specifically expanded Tregs, not effector T cells.
However, expansion remained low compared to polyclonal anti-CD3/CD28 mAb stimulation, although suppressive potential seems to be slightly increased, and future work is needed to optimize growing conditions and obtain sufficient CD8+ Treg numbers for cell therapy.

Altogether, this work improves our understanding of CD8+ Treg recognition and sets the path for development of therapies with peptide epitopes in transplantation.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

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**AUTHOR CONTRIBUTIONS**

E.P. contributed to data collection, experimentation, analysis, and writing of the manuscript. S.B., L.U., J.O., H.H., M.B., K.E., C.U., and K.B. contributed to data collection, experimentation, and analysis. J.R. and S.G. solved the crystal structure and contributed to data collection and experimentation. C.G. conceived, financed, and led the project, analyzed the data, and wrote the manuscript.

**DECLARATION OF INTERESTS**

Patients have been filed based on the results presented in the paper.

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