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Tregitope: Immunomodulation Powerhouse

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ABSTRACT

IVIG is frequently used in the 'pre-conditioning' regimens for higher risk transplants; its effects are attributed in part to induction of Tregs. We have identified regulatory T cell (Treg) epitopes, now known as Tregitopes, in IgG, the main component of intravenous immunoglobulin therapy (IVIg). Tregitopes provide one explanation for the expansion and activation of Treg cells following IVIg treatment. Tregitopes are peptides that exhibit high affinity binding to multiple human HLA Class II DR; they are conserved across IgG isotypes and mammalian species. In vitro and in vivo, for human PBMC and in animal models, Tregitopes activate Tregs. Studies to delineate the mechanism of action have shown that Tregitopes' effects are very similar to IVIg in vitro. Here we demonstrate that Tregitopes induce Tregs to produce IL-10, leading to modulation of dendritic cell phenotype (down-regulation of Class II, CD80 and CD86 and up-regulation of ILT3), and describe the effects of Tregitopes in the ABM-TCR-transgenic skin transplantation model. The discovery of Tregitopes in IgG and other autologous proteins may contribute to improved understanding of the mechanism of action of IVIg and lead to the application of these powerful immunomodulators to improve transplantation success and suppress autoimmune disease, in the future.

1. Introduction

1.1. Tregitopes are highly conserved regulatory T cell peptide epitopes derived from immunoglobulin that induce antigen-specific tolerance

Tregitopes have the following four characteristics: (1) their sequences are highly conserved in similar autologous proteins; (2) they almost all exhibit single 9-mer frames predicted by our EpiMatrix epitope prediction algorithm to bind to at least four different HLA DR alleles and thus are likely to be broadly recognized in the human population; (3) in response to Tregitopes, T cells exhibit a T regulatory phenotype ($CD4^+CD25^+FoxP3^+$); and (4) co-incubation of T cells with Tregitopes and immunogenic peptides inhibits effector T cell (Teff) response to the immunogenic peptides in vitro and suppresses antigen-specific secretion of effector cytokines responses. Tregitope effects have been validated in well-defined animal models: NOD mice (reduction of diabetes incidence, resolution of glucose intolerance) [1]; transplant (induction of Tregs, significant reduction in transplant rejection, and allo-specific tolerance induction); and EAE (suppression of Th17 activation and reduced disease [2]). Here we describe the basic immunobiology of Tregitopes, and the mechanisms by which they induce regulatory T cells to promote tolerance have yet to be elucidated.

1.2. Natural regulatory T cells, Treg epitopes, and tolerance

Autoreactive T cells with moderate TCR affinity may escape deletion in the thymus to circulate in the periphery where they function as 'natural' regulatory T cells (Treg) [3]. These moderate-binding Treg circulate in the periphery where they can suppress immunity against self-antigens. It has become increasingly clear that regulatory T cells, generally characterized as being $CD4^+CD25^+FoxP3^+$, are an important component of immune regulation in the periphery.

1.3. Are Treg epitopes present in self-proteins?

One fundamental question about Treg cells has been their epitope-specificity. Evidence in the literature has been building in support of self-peptides as critical ligands for promoting the

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development of Tregs expressing defined TCRs [4–6]. Among the self-proteins to which tolerance is acutely important for health and homeostasis, immunoglobulins (Ig) are unique. They are present in high concentrations in the plasma and the Ig repertoire's diversity and specificity are directly related to an extremely high rate of somatic hypermutation. The presence of Treg epitopes in the constant regions of the Fv (variable domain) and Fc (constant domains) of immunoglobulin G (IgG) may diminish immune response against the new 'foreign' sequences contained in the complementarity determining regions (CDR) [7]. Thus, after discovering highly promiscuous epitopes in Fv and Fc of IgG, we hypothesized that these epitopes might be Treg epitopes (Tregitopes). While we were making these initial observations, two independent publications appeared to support our hypothesis: Ephrem et al. showed that IVIg induced Tregs in an experimental murine model of multiple sclerosis (EAE) [8] and Anthony and Ravetch demonstrated the importance of antigen-processing pathways for Treg induction by IVIg [9,10].

1.4. Potential for Tregitope therapy?

The importance of Tregs in the control of alloreactive and autoimmune responses and maintenance of tolerance has been noted [11]. Thus, new approaches designed to induce Tregs to be applied for the treatment of transplant rejection, autoimmunity and inflammatory diseases are active areas of investigation. IVIg is one example of a therapy with this effect [12] and evidence is accumulating that Tregitopes provide similar beneficial immunomodulatory effects that in many respects parallel the effects of IVIg.

1.5. Validation of the Tregitope hypothesis

Within our network of Tregitope collaborations, we have performed a number of studies to probe potential therapeutic applications of Tregitopes in mouse models of MS (EAE) [2], cardiac transplant (Najafian et al., unpublished), Type 1 diabetes (T1D, NOD) [1], antigen-induced airway hyper-responsiveness (Mazer et al., unpublished), AAV-mediated gene transfer [13] and others [14]. Together, they show that Tregitopes co-administered with proteins suppress antigen-specific T cell and antibody responses, and induce Treg expansion and function. Side-by-side in vivo comparisons of Tregitope with IVIg have been performed in the autoimmune EAE model (Khoury, Elyaman and DeGroot, unpublished, and [2]) and antigen-induced allergic airway disease (Mazer and DeGroot, unpublished), demonstrating that IVIg effects can be replicated by Tregitope administration. More relevant to transplantation, Tregitopes have been shown to induce adaptive tolerance to test antigens in standard models (T1D, D011.10 [1]). Additional studies by Scott have provided striking evidence that Tregitopes are not antigenic even when formulated in incomplete Freund's adjuvant (IFA) [14].

1.6. Antigen presenting cells (APC) are critical intermediaries between antigens and lymphocytes

APC can include phagocytic cells of the monocyte/macrophage lineage, more specialized dendritic cells (DC), and B cells that in certain contexts have very potent antigen-presenting abilities. APC sample peripheral antigens in the skin, gastrointestinal and respiratory epithelia and migrate to the T cell areas of lymphoid tissue, where they expand and activate antigen-specific helper and killer T cells [15,16]. $CD8^+$ and $CD4^+$ T cells recognize MHC I:peptide and MHC II:peptide complexes, respectively on the surface of APC and initiate adaptive immune responses [17,18].

Beyond their antigen presenting abilities, APC integrate complex soluble and cell–cell contact mediated signals in their milieu so as to coordinate the balance between T cell tolerance and immunity [15,17,19–21]. In addition to expressing MHC molecules on their surfaces, APCs express other cell surface co-stimulatory molecules such as CD80, and CD86 [22–24]. Higher levels of these cell-surface co-stimulatory molecules are expressed following APC activation that together with secretion of pro-inflammatory cytokines and antigen presentation provide highly potent signals for T cell activation [15,25]. The cross-talk between APC and T cells mediated by this complex array of co-stimulatory signals shapes downstream T cell differentiation and activation of effector responses. In contrast, when APC present specific antigens to T cells in the absence of supportive co-stimulation and pro-inflammatory cytokine production, these 'tolerogenic' APC cause cognate T cells to differentiate towards a Treg phenotype.

We have recently developed a comprehensive hypothesis regarding the Tregitope mechanism of action (Fig. 1). We propose that Tregitope-specific T cells are induced when Tregitopes are naturally presented on the surface of APC through degradation of IgG or following escape from the FcRn pathway. Further, we suggest that stimulation of Tregitope-specific Treg cells in the presence of APC may directly down-regulate co-stimulatory signals in the APC, resulting in a tolerogenic phenotype. We hypothesize that presentation of Tregitopes in the context of MHC II to Treg and activation of these Tregitope-specific Tregs are the first steps in the Tregitope mechanism of action, and that these peptides can potentially be exploited to induce tolerance to a wide range of targets including transplanted organs and tissue. Harnessing the ability of Tregitopes to modulate effector T cell responses may lead to new approaches to mitigate auto-reactive T cell responses in a wide range of clinical settings.

2. Materials and methods

2.1. Tregitope and control peptides

Tregitope [26] and control peptides $MOG_{35–55}$ [27] and Flu $HA_{306–318}$ [1] were synthesized by 9-fluoronylmethoxy-carbonyl (Fmoc) synthesis to a purity of >80% as determined by HPLC by New England Peptide (Gardner, MA) and 21st Century Biochemicals (Marlborough, MA). The peptide masses were confirmed using a Q-Star Nanospray Mass Spectrometer. Tregitopes are hydrophobic, thus lyophilized 1 mg aliquots of peptide were reconstituted

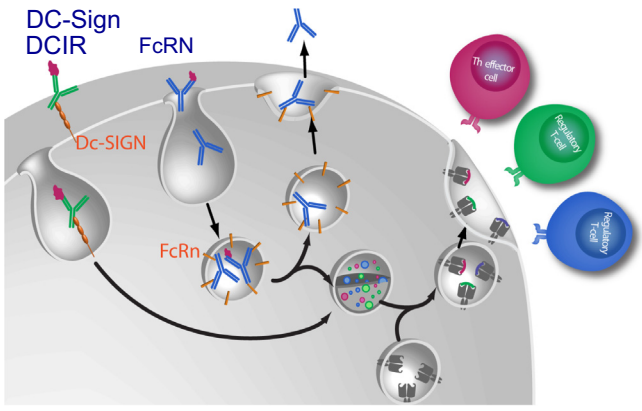

Fig. 1. How does IVIg produce Tregitopes? Many of the effects observed for IVIg parallel the proposed Tregitope mechanism of action. Tregitopes are peptides that would result from IgG internalization and processing, and have been associated with expansion of existing Tregs and induction of iTregs. Internalization of IgG (from IVIg), presentation of Tregitope in the context of MHC Class II, and expansion of Tregitope-specific Tregs would be consistent with recent observations that administration of IVIG induces expansion of Tregs and IL-10 secretion in vivo, in animals and in humans.

by first adding 20 μl of sterile DMSO before bringing up to volume with complete RPMI media for cell culture or 1X PBS for injection. All vehicle controls contained a concentration of DMSO equivalent to the Tregitope peptide formulation.

2.2. Human peripheral blood mononuclear cell isolation

Subjects were recruited under an IRB-approved protocol in collaboration with Clinical Partners LLC (Johnston, RI). PBMC from individual subjects were isolated from whole blood using Ficoll gradient separation. The buffy coat was washed in PBS and resuspended in RPMI containing 10% human AB serum (Valley Biomedical, Winchester, VA), 1% L-glutamine and 0.1% gentamycin for Treg assays.

2.3. Human Treg functional assays

2.3.1. Proliferation measured by CFSE staining

Prior to culture, human PBMCs were labeled with the cell-permeable dye (5-(and -6))-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen, Grand Island, NY) by standard methods [28]. The cells were plated in 24-well culture plates at a density of 2×10^6 viable cells/ml in 2 ml RPMI supplemented with serum and IL-2 under the conditions of no stimulation, MOG_{35-55} as a negative control peptide (self, but not a Tregitope), or human Tregitopes 167 + 289 at a final concentration of 100 μg/ml. On day 3 of culture half of the medium was replaced with fresh RPMI supplemented with serum and IL-2. Cells were harvested on day 7 and stained with CD4, CD25, and FoxP3 as described below to quantify Tregs within the proliferating population.

2.3.2. IL-10. Production

Human PBMCs were plated in 24-well culture plates at a density of 2×10^6 viable cells/ml in 2 ml RPMI supplemented with serum under the conditions of no stimulation, MOG_{35-55} as a negative control peptide, or human Tregitopes 167 + 289 at a final concentration of 100 μg/ml. Cultures were incubated for 72 h total, the last 12 h in the presence of GolgiStop (BD Bioscience, San Jose, CA) to inhibit secretion of cytokine. Upon harvest, cells were stained for surface expression of CD4 and CD25 and intracellular expression of FoxP3 and IL-10 as described below.

2.4. Mice

C57BL/6 female mice were obtained from The Jackson Laboratory and housed in pathogen-free micro-isolator cages at TGA Sciences (Medford, MA) for the duration of these studies. All protocols were approved by the Institutional Animal Care and Use Committee and conducted according to its guidelines. Where indicated, groups of mice were immunized as shown by subcutaneous (SQ) injection in the hind flank with OVA protein emulsified in Complete Freund's adjuvant (CFA, Sigma–Aldrich, St. Louis, MO) on days 0 and 14. Treatments were injected on days 0, 1, 2, 3; 14, 15, and 16. Treatments included a vehicle control containing a concentration of DMSO equivalent to the Tregitope peptide formulation (SQ), mouse (m) Tregitope 167 and 289 peptides formulated together in 1X PBS at 50 μg each per injection (SQ), or 50 mg IVIG (Privigen, Cardinal Health, Dublin, OH) per injection (IP). Whole spleens were harvested on day 17 and processed to generate single-cell lymphocyte populations. Splenic leukocytes were CFSE labeled per standard methods and placed in culture unstimulated or stimulated with 10 μg/ml OVA antigen (Sigma, St. Louis, MO). Cells were cultured for 72 h, harvested, and stained for surface CD4 to determine the percent of OVA-specific CD4 T cell proliferation.

The ABM-tg model [29,30] is a B6/Thy1.2$^+$ TCR-tg mouse model with CD4$^+$ T cells that express a TCR with defined specificity against the bm12 antigen (ABM-tg). These mice were mated with FoxP3 GFP reporter mice to generate ABM-Foxp3GFP TCR-tg mice and the colony was maintained in the Brigham and Women's Animal Facilities [31,32]. Typically, >90% of CD4$^+$ T cells express the tg TCR, and approximately 2% of CD4$^+$ T cells are GFP$^+$. ABM-Foxp3GFP TCR-tg T cells were flow-sorted into GFP$^+$ or GFP$^-$ populations to >98% purity. GFP$^+$ or GFP$^-$ cells (0.6×10^6 cells) were adoptively transferred into congenic WT B6/Thy1.1$^+$ recipient mice. One day later, recipients received skin transplants from C-H-2^{bm12}/KhEg (bm12) donors obtained at the age of 6–8 weeks from the Jackson Laboratory). Recipients were treated with mTregitope 289 or a FluHA negative control peptide on the day of adoptive transfer (100 μg, Day 0), and 50 μg on days 2, 4, and 6. On day 7 following transplantation, draining lymph nodes (dLNs) were harvested from recipient mice and processed into single cell suspensions by standard methods. Proportions of donor bm12-specific Thy1.2$^+$CD4$^+$ T cells that were GFP$^+$ (FoxP3$^+$ Treg) or GFP$^-$ (FoxP3$^-$ effector T cells) were monitored post-transplantation by flow cytometry and distinguished from recipient cells that were Thy1.1$^+$.

2.5. Flow cytometry

Cells were surface or intracellularly stained according to standard protocols [28]. For antigen presenting cell (APC) phenotyping, cells were stained with anti-human CD11c (eBioscience, Clone 3.9), anti-mouse CD11b (eBioscience, Clone M1/70), anti-human HLA-DR, DP, DQ (BD Biosciences Clone Tu39), anti-mouse pan-MHC II (eBioscience, Clone M5/114.15.2), anti-human CD80 (BioLegend, Clone 2D10), anti-mouse CD80 (BioLegend, Clone 16-10A1), anti-human CD86 (BioLegend IT2.2), anti-mouse CD86 (eBioscience, Clone GL1), and human anti-ILT3 (eBioscience, Clone ZM4.1). Human Treg cells were identified by the combination of anti-human CD4 (eBioscience, Clone OKT4), anti-human CD25 (eBioscience, Clone BC96), and anti-human FoxP3 (eBioscience, Clone 236A/E7). Treg functions were studied by combining this panel with CFSE (Invitrogen) for proliferation or with anti-human IL-10 (eBioscience, Clone JES3-9D7) for cytokine production. OVA-specific effector T cells were surface stained with CD4 (eBioscience RM4-4) to determine the proportion of CD4 T cells that proliferated (CFSE low) in response to OVA stimulation ex vivo. Cells were acquired on a FacsCalibur Flow Cytometer equipped with an optional argon laser and CellQuest software (Becton Dickenson, San Jose, CA), and analyzed using FlowJo Software (Tree Star, Ashland, OR).

3. Results

3.1. Tregitopes stimulate Treg proliferation

Previous studies had demonstrated increased proportions of FoxP3$^+$ Tregs within the CD4$^+$CD25$^+$ population upon Tregitope exposure [26]. To expand upon these findings, we set out to determine whether Tregitopes stimulated Treg proliferation. Human PBMCs were labeled with CFSE dye before culture with media, 100 μg/ml negative control peptide (MOG_{35-55}), or 100ug/ml hTregitopes 167 + 289 for 7 days in the presence of 10 ng/ml IL-2. At the conclusion of the culture, cells were stained for intracellular expression of FoxP3 [33] and surface expression of CD4 and CD25. CD4$^+$ T cells that had divided were identified by flow cytometry as those exhibiting dilution of CFSE dye; these were analyzed for CD25 and FoxP3 (Fig. 2). Among those CD4 T cells that had proliferated, Tregitope-exposed cultures exhibited increased

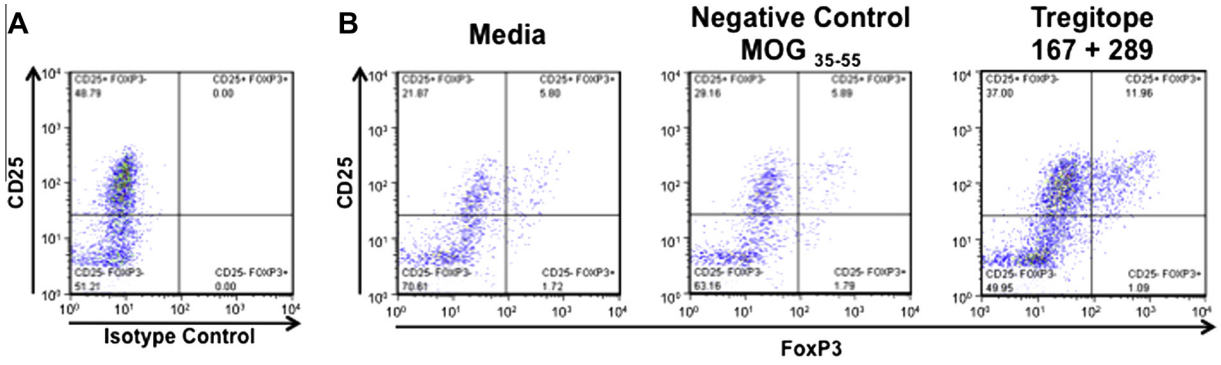

Fig. 2. Tregitopes stimulate Treg proliferation. Human PBMCs were labeled with CFSE and incubated with media alone, control MOG_{35-55} (100 μg/ml), or hTregitopes 167 + 289 (100 μg/ml total) in the presence of IL-2. Cells were fed fresh media + IL-2 on day 3 and harvested on day 7. Cells were stained for CD4, CD25, and FoxP3. The gating strategy was based on $CD4^+$ T cells that were CFSE low, indicating proliferation, then analyzed for CD25 vs isotype control (A) or CD25 vs FoxP3 (B). The proportions of proliferating CD4 T cells that express the Treg phenotype ($CD25^+FoxP3^+$, upper right quadrant) are indicated; increases in proliferating Treg were observed with Tregitope (right) as compared to either media (left) or MOG peptide (center) controls.

proportions with a Treg ($CD25^+FoxP3^+$) phenotype when compared to media- or control peptide (Fig. 2B). These data support the hypothesis that MHC Class II-binding Tregitopes stimulate Tregitope-specific $CD4^+$ T cells with surface molecules consistent with a Treg phenotype to proliferate.

3.2. Tregitope-mediated induction of IL-10

One attribute of activated Treg demonstrated by this group and others is the production of IL-10 [26,34,35]. Therefore we asked whether Tregitope exposure led Tregs to express this key cytokine associated with tolerance induction. Human PBMCs were incubated with media alone, MOG_{35-55} peptide (100 μg/ml), or hTregitopes 167 + 289 (100 μg/ml). Cells were incubated for 72 h, the last 18 h in the presence of the protein transport inhibitor GolgiStop, Cells were harvested and subjected to intracellular staining for IL-10 together with FoxP3 and surface staining for CD4 and CD25. Flow cytometric analysis was used to gate on the $CD4^+CD25^+$ cells (data not shown) within which the $FoxP3^+$ Treg population could be examined for IL-10 expression. When the $CD4^+CD25^+$ T cells were examined for expression of IL-10 and FoxP3, it was confirmed that Tregitope exposure increased the proportion of Treg that expressed IL-10 (Fig. 3).

3.3. Effects of Tregitopes on APC phenotype

CD80, CD86, and MHC Class II expression by APC are critically important contributors to the balance of Treg and T effector cell responses. Moreover, evidence in the literature indicated that these co-stimulatory molecules were down-modulated in response to a Tregitope-like peptide [36] and IVIG [37]. We therefore measured the expression of these surface molecules after Tregitope treatment, postulating that their modulation might contribute to the mechanism(s) by which Tregitopes induce tolerance. Mouse splenic leukocytes or human PBMCs were incubated with media alone, a negative control peptide, or Tregitopes. Cells were collected at 24, 48, or 72 h for surface marker staining and flow cytometry analysis. In the example shown here, mouse splenic APCs ($CD11b^+$) incubated with murine Tregitopes 167 + 289 for 72 h exhibited decreased expression of MHC II (46% decrease), CD80 (59% decrease) and CD86 (36% decrease) as compared to cells incubated with media alone (Fig. 4). As an additional negative control, cells were incubated with peptide that also binds to murine MHC. Neither control (neither media nor $Flu_{306-318}$ peptide) caused decreases in CD80, CD86 or MHC II. The shift towards a tolerogenic phenotype was only observed when murine (Fig. 4) or human PBMCs (data not shown) were incubated with respective murine or human Tregitopes 167 + 289 for 72 h. Moreover, these changes were not evident at 24 or 48 h, suggesting that the shift in the APC phenotype was secondary to other, primary Tregtiope-mediated events taking place in the culture, such as the activation of Tregs as described in Sections 3.1 and 3.2 above.

As further confirmation that Tregitope exposure was skewing the phenotype of APCs in these cultures, we examined expression of a marker that has been positively associated with tolerance induction. ILT3 is a receptor expressed by APCs that has been shown to negatively regulate activation and can be used for antigen uptake [38]. In human APC, intracellular signaling

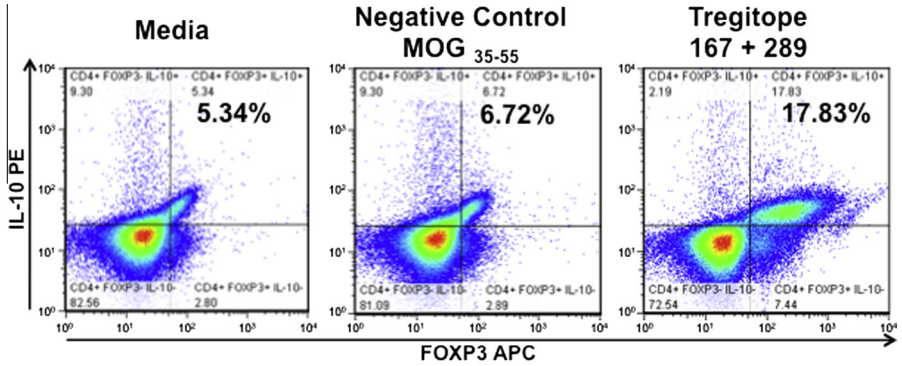

Fig. 3. Tregitopes stimulate IL-10 in Tregs. Human PBMCs were incubated for 72 h with media, control MOG_{35-55} (100 μg/ml), or hTregitopes 167 + 289 (100 μg total). Cells were stained for intracellular IL-10 and T cell markers CD4, CD25, and FoxP3. After gating on $CD4^+CD25^+$ cells, dot-plots display $\%IL\text{-}10^+$ $FoxP3^+$ cells (upper right quadrant). Increased proportions of $IL\text{-}10^+$ Treg were observed upon Tregitope exposure (right) in contrast to media (left) or MOG (center).

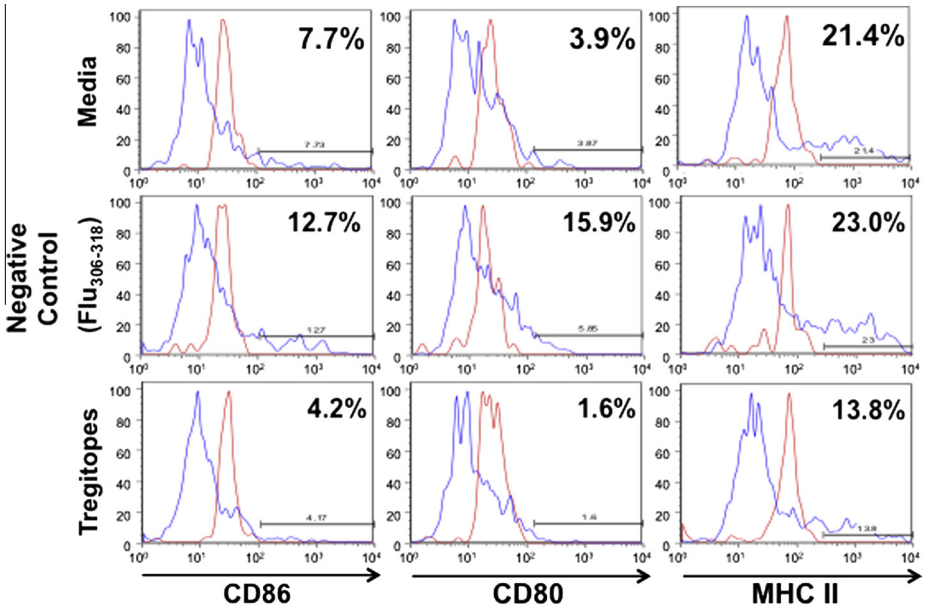

Fig. 4. Effects of Tregitopes on APC Phenotype. Mouse splenic leukocytes were incubated with media alone, $Flu_{306-318}$ at 100 μg/ml as control, or mTregitopes 167 + 289 at 100 μg total. Cells were harvested after 72 h and stained for the APC marker CD11b, and the costimulatory markers CD86, CD80, and MHC II. Histograms depict surface expression of CD86, CD80, and MHC II on Tregitope-exposed CD11b populations (blue, isotype control in red); the percent positive within the selected region is indicated in the upper right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

downstream of ILT3 inhibits NF-kappa B activation and the transcription of costimulatory molecules to promote anergy and regulatory function in T cells with cognate specificity [39]. Human PBMCs were cultured for 72 h with media alone, MOG_{35-55} peptide (100 μg/ml), or human Tregitopes 167 + 289 (100 μg/ml). At the conclusion of the culture, cells were collected and stained for CD11c and ILT3. Examination of ILT3 on the surface of the $CD11c^+$ population indicated that expression remained low under conditions of media alone or MOG_{35-55} negative control, but there was a measurable induction in the presence of Tregitopes (Fig. 5). These results provided further evidence that Tregitopes, either directly or indirectly, were promoting a tolerogenic APC phenotype.

3.4. Tregitopes recapitulate effects of IVIg to suppress antigen-specific T cell responses

To further support our hypothesis that Tregitopes promote tolerance induction like the known immunomodulator IVIg, we sought to directly compare their ability to modulate in vivo antigen-specific T cell responses. Thus we established a basic model of OVA immunogenicity adapted from Aubin et al. in which administration of OVA antigen elicits a robust, systemic specific T cell response that is suppressed in mice administered IVIg [40]. To stimulate OVA-specific responses, mice were administered OVA formulated in CFA by subcutaneous (SQ) injection on days 0 and 14. Mice were treated on days 0, 1, 2, 3; 14, 15, and 16 with vehicle control (SQ), mouse Tregitope 167 and 289 peptides formulated together in saline at 50 μg each per injection (SQ), or 50 mg IVIg per injection (IP). Splenic leukocytes were harvested on Day 17, CFSE labeled, and cultured in the presence of whole OVA Ag for 72 h. At the conclusion of the culture, cells were harvested and co-stained for CD4. After gating on the $CD4^+$ T cells, the proportion of CFSE low cells within the $CD4^+$ subset was used to quantify the proportion of CD4 T cells that proliferated in response to OVA Ag ex vivo. Averages of 12 mice per group pooled from three individual experiments are shown ± standard error (Fig. 6). In mice treated with IVIg, OVA-specific CD4 T cell proliferation was decreased

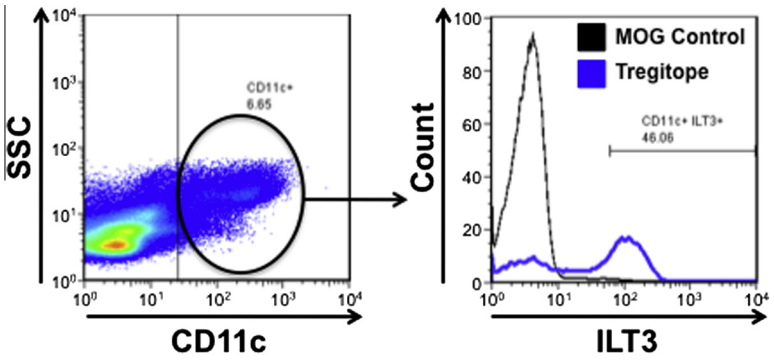

Fig. 5. Tregitope-mediated induction of ILT3. Human PBMC were incubated for 72 h with media, control MOG_{35-55} (100 μg/ml), or hTregitopes 167 and 289 (100 μg total). Cells were stained for CD11c and ILT3. After gating on $CD11c^+$ cells (left), histograms were derived that display the proportion of $ILT3^+$ cells within the $CD11c^+$ population. Increased proportions of $ILT3^+$ APCs were observed upon Tregitope exposure (blue) relative to MOG (black). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

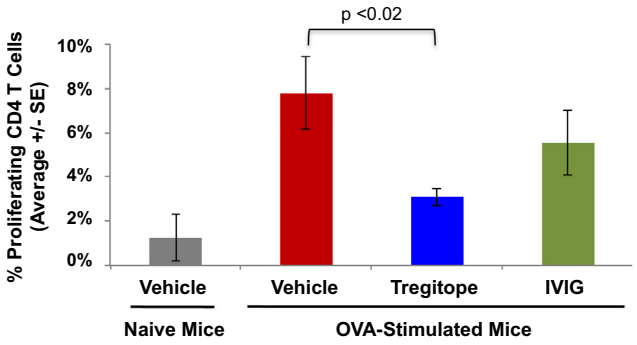

Fig. 6. Tregitope exposure in vivo suppresses antigen-specific T cell proliferation ex vivo. Splenic leukocytes from mice immunized with OVA and treated with vehicle, Tregitope, or IVIg were harvested on Day 17, CFSE labeled, and cultured in the presence of whole OVA Ag for 72 h. Cells were harvested and co-stained for CD4. After gating on the $CD4^+$ T cells, analysis of CFSE low cells indicates the proportions of CD4 T cells that proliferated in response to OVA Ag ex vivo. Averages of 11–12 mice per group pooled from 3 individual experiments are shown ± standard error. Group averages were compared using a Student's *t*-test.

relative to vehicle control, recapitulating the original Aubin experiment [40]. As postulated, Tregitope treatment also resulted in the significant inhibition of OVA-specific CD4 T cell proliferation as compared to vehicle-treated mice. The magnitude of the IVIg and Tregitope effects on CD4 T cell proliferation was comparable, however the effective dose of Tregitope to achieve this effect was significantly decreased: Tregitopes were administered as a 50 μg/injection relative to IVIg that was administered at 50 mg/injection.

3.5. Tregitopes promote expansion of alloreactive Tregs and induce graft-reactive iTregs in vivo

The capacity of Tregs to modulate adaptive and innate immune responses has led to their investigation for use in novel strategies to regulate allogeneic T cell responses. We believe that Tregitopes, by expanding Tregs, are capable of regulating alloimmune responses. This hypothesis was tested in mice transgenic for a TCR specific for bm12 antigen crossed with mice expressing GFP under control of the FoxP3 promoter (ABM-Foxp3GFP) [31,32]. In these mice, typically >90% of $CD4^+$ T cells express the transgenic TCR, and approximately 2% of $CD4^+$ T cells are GFP^+. Moreover, ABM-Foxp3GFP $CD4^+$ cells express CD90.2 ($Thy1.2^+$) to distinguish them from recipient CD4 T cells that are $Thy1.1^+$. Donor ABM-Foxp3GFP CD4 T cells were isolated by flow cytometry, sorting for GFP^+ or GFP^- populations with >98% purity. These $Thy1.2^+$-$FoxP3^+GFP^+$ or $Foxp3^-GFP^-$ cells (0.6×10^6) were adoptively transferred into Thy1.1 B6 recipients 1 day prior to bm12 skin transplantation. Recipients were treated with Tregitope 289 or a FluHA negative control peptide on the day of adoptive transfer (100 μg, Day 0), and 50 μg on days 2, 4, and 6 (Fig 7A). In Tregitope-treated mice that received $CD4^+Thy1.2^+GFP^+$ donor Treg cells, significant increases in the $CD4^+Thy1.2^+GFP^+$Treg populations were observed relative to controls (Fig 7B) indicating that transferred $FoxP3^+$ Tregs were expanding. For Tregitope-treated recipients of $CD4^+Thy1.2^+GFP^-$ cells, increased numbers of $CD4^+Thy1.2^+GFP^+$ populations (Fig 7C) demonstrate that the ABM-Tg $CD4^+Thy1.2^+$ GFP^- cells that were transferred were induced in vivo to express FoxP3, suggesting the induction of antigen-specific Tregs in the presence of Tregitope. Moreover, Tregitope-treated mice exhibited fewer donor-derived $FoxP3^-$ Teffector-memory cells ($Thy1.2^+CD4^+$ $CD44^{high}CD62L^{low}$) than controls (Fig 7D) indicating that Tregitope treatment was associated with suppression of ABM12-specific T cell responses. Taken together, these data indicate that Tregitopes promote expansion of Tregs as well as induce adaptive Tregs.

4. Discussion

We present here direct evidence that upon MHC II-mediated presentation to Tregs, Tregitopes stimulate the activation of Tregs as demonstrated by proliferation and IL-10 production (Figs. 2 and 3), promote a tolerogenic APC phenotype (Figs. 4 and 5), and modulate antigen-specific effector T cell responses in vivo (Figs. 6 and 7). These data support our working hypothesis for the mechanism of action (Fig. 8) whereby Tregitope effects are initiated upon APC/MHC II-mediated presentation to a population of regulatory T cells specific for self-IgG-derived epitopes. Tregs that recognize Tregitopes presented in the context of MHC II become activated, produce IL-10, and interact with the APC to reinforce the development of a tolerogenic phenotype. These tolerogenic APC and/or Treg act on local antigen-specific Teff to suppress their effector responses.

4.1. Tregitopes may provide a safer alternative to IVIG

Many adverse effects (AEs) associated with IVIG administration are mild and transient. Though rare, more severe AEs include acute renal failure, thromboembolic events, skin-related effects including toxic epidermal necrolysis and aseptic meningitis [41,42]. Patients also report generalized asthenia, nausea and vomiting. Poor tolerance of weekly IVIg infusions limits its use in some autoimmune diseases [43]. In general however, IVIg is considered relatively safe. Thus the main incentives to seek alternatives to IVIg are to conserve the limited supply for patients with primary immunodeficiencies for whom this is the only option, and to reduce adverse events associated with IVIg treatment. Since Tregitopes replicate the tolerogenic effects of IVIg in vitro and in vivo where those effects are Treg-mediated, Tregitopes might serve as a safe therapeutic alternative to IVIG for certain conditions, like induction of transplant tolerance and suppression of autoimmunity.

4.2. Tregitope applications in transplant

Organ transplantation remains the therapy of choice for the majority of patients with end-stage organ failure. Traditional immunosuppressive regimens rely on agents which cause general suppression of the adaptive immune response and are consequently associated with various undesirable related side-effects including opportunistic infections and malignancies. The achievement of robust and reproducible transplantation tolerance by clinically applicable and safe therapeutic regimens is, therefore, a major goal in transplantation research. The discovery of Tregitopes in IgG may allow clinicians to safely harness the therapeutic potential of Tregs to modulate immune responses while avoiding administration of immunoglobulin therapy. They have vast potential as a unique and novel pro-tolerogenic therapy that, used alone or in combination with costimulatory blockade, may induce tolerance in the context of transplantation.

4.3. Additional Tregitope applications

Replacement of a pooled-donor, blood-derived product for which the mechanism of action is not certain with a therapeutic product for which the Active Pharmaceutical Ingredient (API) is precisely defined, would represent a major step forward for the field of autoimmune disease therapy. If the induction of regulatory T cells by IVIG can be attributed to Tregitopes, then a number of autoimmune diseases for which IVIG therapy is currently used, on-label and off, may be appropriate targets for immunomodulatory formulations that only contain Tregitope peptides. Two examples of disease in which IVIG is the predominant immunotherapy

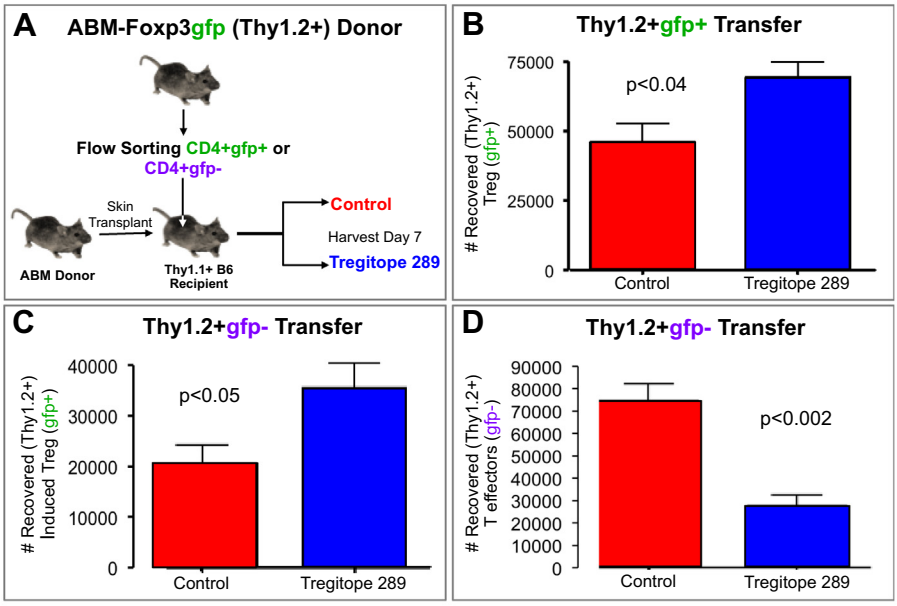

Fig. 7. Tregitopes promote expansion of antigen-specific Tregs in vivo. Thy1.2^{+}FoxP3^{+} GFP^{+} or Foxp3^{-}GFP^{-} cells from ABM-FoxP3GFP mice (0.6×10^{6}) were adoptively transferred into Thy1.1 B6 recipients 1 day prior to bm12 skin transplantation. Recipients were treated with Tregitope 289 or negative control peptide on the day of adoptive transfer (100 μg, Day 0), and 50 μg on days 2, 4, and 6 as depicted (A). Mice that received CD4^{+} Thy1.2^{+} GFP^{+} Treg cells were analyzed for numbers of CD4^{+} Thy1.2^{+} GFP^{+} Treg populations after Tregitope and or control treatments (B). Alternatively, Tregitope- or control-treated mice that received CD4^{+} Thy1.2^{+} GFP^{-} cells were analyzed for numbers of CD4^{+} Thy1.2^{+} GFP^{+} Treg (C) or Thy1.2^{+} CD44^{high}CD62L^{low} GFP^{-} effector T cell (D) populations. Results are expressed as mean ± standard error; groups were compared using a Student's *t*-test.

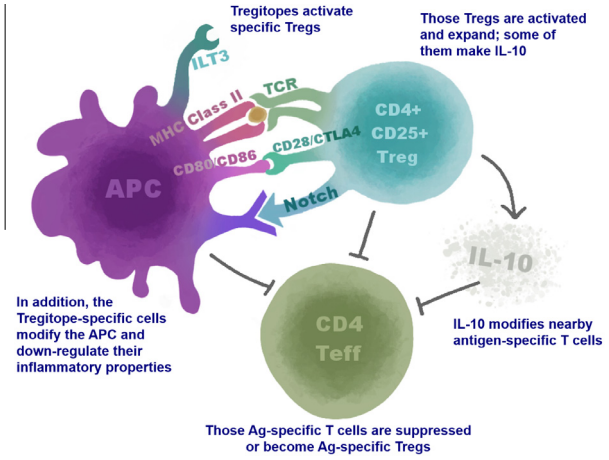

Fig. 8. Proposed Tregitope mechanism of action.

include Chronic Inflammatory Demyelinating Polyneuropathy (CIDP) and Multifocal Motor Neuropathy (MMN). Introduction of Tregitopes as alternatives to IVIG would also have a dramatic impact on the demand for IVIG for immune modulation therapy [9,44,45]. IVIG also acts rapidly and effectively in Immune Thrombocytopenic Purpura (ITP), Kawasaki Syndrome (KS), polymyositis, dermatomyositis, neurological syndromes such as Guillain–Barré and CIDP, cases of severe steroid-dependent asthma, and many others [44,45]. Tregitope-peptide therapy may be an attractive alternative the systemic immune suppression treatments that are sometimes used for those conditions.

4.4. Potential for Tregitope-mediated antigen-specific tolerance induction

As Tregitopes appear to be able to induce antigen-specific tolerance (induced Tregs that are specific to co-administered proteins), the possibility of tailoring the Tregitope therapy to specific autoimmune diseases would represent an additional advantage for clinical applications. One could envision allergen-specific treatments using combinations of Tregitopes and allergen proteins or peptides, and treatment for autoimmune diseases such as diabetes, that would rely on co-administration of Tregitope and diabetes antigens. The idea of specifically generating MOG-reactive Treg cells is also particularly attractive for the therapy of MS where, based on our data in the EAE mouse model, we believe that Tregitopes induce antigen-specific adaptive tolerance. Tregitopes may have even broader applications in protein therapeutics, animal health, and blood factor or enzyme replacement therapy.

4.5. Moving Tregitope towards the clinic

Treatment of many autoimmune diseases relies on immunosuppressive therapy rather than on treatments directed toward restoring a balance between effector and regulatory immune responses. Given that Tregitope peptides appear to induce adaptive tolerance in a mouse model, the next consideration is to evaluate the optimal formulation and determine the best time course of the tolerance induction, in addition to measuring the duration of response, the dose required, the safety and toxicity of the treatment (vis-à-vis other immune responses) and optimal formulation/route of administration. Our preliminary studies suggest that adaptive tolerance induction may be within reach, raising hopes that we may be on the right path for the development of an effective immunotherapy based approach to autoimmune disease.

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