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Engineered MBP-specific human Tregs ameliorate MOG-induced EAE through IL-2-triggered inhibition of effector T cells

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Abstract

Expanded polyclonal T regulatory cells (Tregs) offer great promise for the treatment of immune-mediated diseases. Inhibition by Tregs is under the control of the T-cell receptor (TCR). Therefore, we created Tregs with defined antigen specificity, using a recombinant T-cell receptor isolated from a myelin-basic protein specific T-cell clone of a multiple sclerosis (MS) patient (Ob2F3). We expressed this TCR using a retroviral expression vector in human Tregs from peripheral blood. We observed that transduced Tregs were activated *in vitro* in response to myelin basic protein (MBP) peptide on DR15 antigen-presenting cells (APC) and upregulated Treg markers, Foxp3, LAP and Helios. These engineered MBP-specific Tregs could suppress MBP-specific T effector cells, and were also able to suppress T cells with other specificities after Tregs had been activated through the TCR. Importantly, we showed that these engineered Tregs were able to function effectively in the presence of strong TLR-induced inflammatory signals, and that MBP-specific Tregs ameliorated EAE in myelin oligodendrocyte glycoprotein (MOG)-immunized DR15 transgenic mice. We further demonstrated *in vitro* that IL-2 produced by neighboring effector T cells activated MBP-specific Tregs, initiating contact-independent suppression to T effectors in local milieu. Mechanistic studies demonstrated that bystander suppression *in vivo* may involve transfer of soluble mediators, enhanced by cell contact between Tregs and effectors. Taken together, we show that engineered clonal MBP-specific Tregs are able to suppress autoimmune pathology in EAE. This approach may serve as a cellular therapy for MS patients with the common DR15 haplotype that is associated with disease susceptibility.

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Author Contributions

YCK designed the study, constructed Ob2F3 TCR, expanded all engineered T effectors and Tregs, conducted *in vitro* experiments, and wrote the paper. AZ performed *in vivo* experiments. JY and WEC performed the analyses of immunostaining and H&E staining, and JRL provided additional analysis and edited the manuscript. KWW provided sequence information of MBP-specific TCR and advised the construction of TCR. DWS supervised this study and wrote the paper.

Introduction

Regulatory T cells (Tregs) have been proposed as a potential clinical therapy for variety of adverse immune disorders, including autoimmune diseases and the development of anti-drug antibodies. However, polyclonal Tregs encompass multiple specificities and could potentially be globally immunosuppressive (1). Based on the pioneering work by June and colleagues using chimeric antigen receptors (CAR) to redirect the specificity of cytotoxic cells in cancer (2) and of Eshhar *et al.* using “T-bodies” in autoimmunity (3, 4), we previously developed an approach to render polyclonal Tregs “specific” by transducing them with T-cell receptor (TCR) V genes from a patient’s T-cell clone specific for a known epitope in clotting factor VIII (5, 6). These Tregs were highly effective at suppressing both T and B cell responses to this cognate antigen *in vitro* and *in vivo*, including the diminution of adverse antibody formation. Herein, we further examined this approach for potential application in multiple sclerosis (MS) by expressing a myelin basic protein (MBP)-specific TCR derived from a MS patient in human Tregs.

Multiple sclerosis (MS) is a neurodegenerative autoimmune disorder, in which pathogenic autoreactive T cells migrate into the central nervous system across the blood-brain barrier (BBB) to attack myelin sheaths directly via cytokines and indirectly by the hyper-activation of antigen-presenting cells (APCs), resulting in destruction of the myelin sheath and causing severe impairment of neuronal function. For the last two decades, there have been several conceptual trials with Treg infusions for the prevention or reversal of experimental allergic encephalomyelitis (EAE), a mouse model of MS. However, most Treg infusions were produced from polyclonal CD25+ve natural Tregs (7) or polyclonal induced Tregs (8). In one report, specific CD25+ve Tregs were isolated from MBP-specific TCR-transgenic mice; these Tregs suppressed MBP-induced EAE mice but did not ameliorate MOG- or proteolipid protein (PLP)-induced EAE (9). These conceptual approaches were required to understand the immunological activity of infused Tregs in autoimmunity *in vivo* but do not promise therapeutic potential in clinical practice in MS because pathogenic T cells with multiple specificities could engage in demyelination and identification of pathogenic epitopes in individual patients is problematic. Hence, *in vitro* and *in vivo* approaches that utilize bystander suppressive effects from single clonal engineered Tregs are still required.

In this report, we demonstrate that Treg cells from normal donors can be made highly reactive to MBP by expressing an MBP-specific recombinant TCR, and that these MBP-specific single cloned Tregs not only specifically suppress T effectors of the same antigen-specificity but also bystander T effectors, even with Toll-like receptor-induced strong inflammatory conditions *in vitro*. Transfer of MBP-specific human Tregs into MOG peptide-immunized recipients also led to amelioration of EAE through bystander suppression *in vivo*. We further found that IL-2 from locally activated T effectors is critical for engineered Tregs to suppress the local T effectors with different specificity via soluble suppressive factors. Thus, we provide proof of principle for *in vivo* efficacy of MBP-specific engineered Tregs via a bystander suppression mechanism for the first time as a potential therapy for MS.

Results

Transduction of primary human T cells with a retroviral vector driving expression of TCR specific for myelin basic protein (85-99)

We pre-stimulated naïve CD4⁺ T cells (CD4⁺CD25⁻CD127^{hi}CD45RA⁺) from healthy donor's PBMCs and retrovirally transduced them with the DR15-restricted MBP-specific recombinant TCR, called Ob2F3 (10, 11). To confirm that the transduced recombinant TCR continues to recognize MBP, Ob2F3 TCR-expressing effector T cells (Ob2F3 T effectors) were labeled with cell proliferation dye eFluor 450 (CPD450) and then co-incubated with γ -irradiated HLA-DR1/DR15 PBMCs in the presence of MBP85-99 or OVA23-339 without rIL-2 for 4–5 days, followed by flow cytometric analysis of CPD450 dilution. Stimulation of these Ob2F3 T effector cells by MBP85-99 is reflected by the dilution of proliferation dye and an increase in V β 2⁺ T cells in the GFP-positive transduced cells. No proliferation of GFP-positive Ob2F3 cells was detected without the addition of the MBP peptide, indicating that Ob2F3 TCR does not recognize non-specific autologous peptides bound to the HLA-DR15 complex (data not shown). This demonstrates acquisition of specificity toward MBP (Supplemental figure 1). Since the PBMC donors are not previously HLA-typed, we also observed variable donor-dependent proliferation of GFP-negative T cells, which we believe to be due to allorecognition of the DR1/DR15 antigen-presenting cells.

MBP-specific engineered Tregs upregulate activated regulatory T-cell markers upon stimulation with MBP85-99 and suppress MBP-specific proliferation *in vitro*

To express Ob2F3 TCR in human Tregs, CD4⁺CD25^{hi}127^{lo/-} cells were sorted by flow cytometry, and then pre-stimulated with anti-CD3 ϵ and anti-CD28 antibodies in the presence of IL-2 for 48 to 72 hours, followed by retroviral transduction of Ob2F3 TCR in the presence of ODNps25 (12). For expansion *in vitro*, transduced cells were incubated for an additional 7 days in the presence of IL-2, and then cells were sorted for GFP⁺ cells by flow cytometry to maintain the purity of transduced Ob2F3 Tregs during subsequent *in vitro* culture. To confirm that the Ob2F3 Tregs retained regulatory T cell markers, after a 3 to 4-week expansion of Ob2F3 Tregs, the expression of Foxp3 and Helios was examined by flow cytometry after 48 hr-activation with MBP85-99 plus DR1/DR15 APC in the presence of IL-2. GARP and LAP were also measured as activated Treg markers. As shown in Figure 1, the majority of expanded GFP-expressing Ob2F3 Tregs (77.7%) markedly retained expression of Foxp3 and Helios. Moreover, these cells did not secrete IFN γ or IL-2, as opposed to expanded T effector cells, which were positive for both cytokines (Fig. 1A). As expected, Ob2F3 Tregs specifically upregulated the expression of LAP and GARP on their cell surface after co-culture with MBP85-99 and APCs (Fig. 1B). There was no induction of LAP and GARP without the addition of peptide, as expected (data not shown).

To test for their ability to suppress MBP-specific effector cells, Ob2F3 T effector cells were co-cultured with Ob2F3 Tregs or 17195 Tregs (specific to coagulation factor VIII) at various ratios either with MBP85-99 or anti-CD3 ϵ ; cell proliferation of the effector cells was measured as above after 3–5 days (5) using [³H]-thymidine incorporation. As shown in Figure 1C, Ob2F3 Tregs suppressed the proliferation of the MBP-specific effector cells even when the effector cells were in 4–8-fold excess, whereas non-specific 17195 Tregs only

suppressed partially at high Treg ratios (1:1 and 2:1). In contrast, anti-CD3e stimulation led to equal suppression in the both specific and mock Tregs co-cultures (data not shown). Thus, Ob2F3 Tregs are highly effective at suppressing proliferation and cytokine production (Supplemental figure 2) of MBP-specific T effector cells in the presence of specific antigen.

MS is an inflammatory disease. Therefore, it is important to evaluate whether specific Tregs are effective in the presence of strong inflammatory signals. To mimic inflammation, we added different TLR agonists to the co-cultures. These TLR agonists enhanced the proliferation of effector T cells to MBP in the absence of specific Tregs. However, despite the strong TLR stimuli, Ob2F3 Tregs still suppressed the proliferation of the effector cells down to the baseline levels (Supplemental figure 3). Taken together, we concluded that Ob2F3 Tregs retained functional Treg phenotypes and specifically suppressed the proliferation of MBP-specific T effectors even under strong inflammatory conditions.

MBP-specific engineered Tregs can exhibit bystander suppression locally in vitro

Lymphocytes from MS patients can respond to multiple CNS antigens. To test whether Ob2F3 Tregs could suppress the response to additional antigens, we performed a bystander suppression assay by co-culturing with T effectors transgenic for a TCR specific for a non-CNS antigen (17195), that recognize a FVIII peptide in the C2 domain, p2191–2210 (pC2) restricted by HLA-DRB1*0101 (DR1). Briefly, CPD450-labeled 17195 T effector cells were co-cultured with Ob2F3 Tregs at the indicated ratios with γ -irradiated HLA-DR1/DR15 PBMCs as APC for 3–5 days in the presence of MBP 85-99 (pMBP) and FVIII p2191–2220 (pC2) together or FVIII pC2 alone without the addition of rIL-2, followed by counting of CPD450-labeled effector cells by flow cytometry. Ob2F3 Tregs suppressed the proliferation of the 17195 T effectors significantly only when MBP85-99 was present to activate the Tregs. The efficacy of this bystander suppression was equivalent to standard suppression by 17195 Tregs (Fig. 2). The result suggests that single clonal MBP-specific Tregs are capable to suppress effector T cells with a different antigen specificity when both antigens are presented by the same APC.

MBP-specific engineered human Tregs ameliorate MOG-induced mouse EAE via bystander suppression in vivo

To further demonstrate bystander suppressive function of Ob2F3 Tregs *in vivo*, we employed the MOG-induced experimental allergic encephalomyelitis (EAE), an animal model for MS, in human HLA DR15 transgenic mice. We administered human Ob2F3 Tregs intravenously on day 7 after active immunization with MOG35-55 in complete Freund's adjuvant, at a time when the CNS inflammation was already well established. Although these cells were xenogeneic to the murine hosts, Ob2F3 Tregs significantly reduced mean and cumulative disease scores in MOG-induced EAE mice compared to PBS and 17195 Treg-infused mice (Fig. 3A and 3B). Histological analysis confirmed CNS migration of Ob2F3 Tregs in brain and spinal cord 2 days after infusion (Fig. 3C); 17195 Tregs were detected to have migrated to a lesser extent on the same day. Importantly, the intensity of hCD4-PE was significantly higher in the both tissues of Ob2F3 Treg-infused mice than of non-specific 17195 TCR Treg-infused mice (Fig. 3C). On day 17, the infused Ob2F3 or 17195 human Tregs were no longer apparent presumably due to xenorecognition in either brain or spinal cord tissues.

Furthermore, histopathologic analysis of the spinal cord indicated that Ob2F3-treated mice showed reduced inflammation compared to 17195 or PBS groups on day 17, consistent with the decreased EAE scores observed in Figure 3A (Fig. 3D). To confirm the possible accumulation of infused human Tregs, we further stained the slides with anti-human CD4 and anti-mouse CD4 antibodies: the perivascular cells were primarily mouse CD4 T cells (data not shown). From these *in vivo* results, we concluded that single clonally focused MBP-specific Ob2F3 Tregs can infiltrate into inflamed CNS tissues, and ameliorate the pathogenesis of MOG-induced encephalomyelitis via bystander suppression mechanism in the local milieu (13).

Contactless bystander suppression by MBP-specific Tregs requires the co-activation of neighboring T effectors

MBP-specific engineered Tregs successfully demonstrated bystander T effector cell suppression *in vitro* and under pathogenic conditions *in vivo*. However, how such bystander suppression is generated in physiological inflammatory conditions was not clear. To address this, we first mimicked bystander suppression of T effectors across different APCs to determine whether inter-APC bystander suppression would occur. For inter-APC stimulation, γ -irradiated HLA-DR1/DR15 PBMCs were used to have both restricting elements present. Cells were pulsed with MBP p85-99 and FVIII pC2 2191–2220 together or separately, then co-localized in same culture well as shown in figure 4A. CPD-450 labeled 17195 T effectors and Ob2F3 Tregs were co-cultured for 4–5 days, followed by cell counting of CPD450-labeled cells by flow cytometry (Fig. 4A). As in Figure 2, Ob2F3 Tregs strongly suppressed bystander 17195 T effectors (**part II in Fig. 4A**) that shared the same APC. Importantly, Ob2F3 Tregs also significantly suppressed bystander 17195 T effector stimulated separately on different APCs as well (**part III in Fig. 4A**), and this bystander suppression across APC was retained even in the presence of extra T effectors in same APC conditions. This indicates that bystander suppression by single clonal engineered Tregs was not limited cells interacting with the same APC.

We next addressed whether the bystander suppression across APC could be accomplished in a contactless manner. Ob2F3 Tregs with antigen-loaded APCs were placed in one well and Ob2F3 T effectors with same APCs in next well (**part I in Fig. 4B**), then created ~1 mm holes in 96-well culture plate between these two wells to allow fluids and presumably soluble factors but not cells to be transferred horizontally (**part II and III in Fig. 4B**). As usual, suppression occurred when Tregs and effectors were co-cultured in the same well (Fig. 4C, **part IV**). When specific Tregs and T effectors were separately activated, no suppression occurred (Fig. 4, **part II**). However, when Tregs were activated with T effectors together in one well, then the T effectors in the adjacent well were significantly suppressed (Fig. 4B, **part III**). These data suggested that proximal activation of T effectors enabled Tregs to suppress “contactless” bystander T effectors at a distance.

Initial IL-2 produced by neighboring T effectors enables MBP-specific Tregs to suppress contactless bystander T effectors

IL-2 is a dominant cytokine produced by T effectors during an initial activation stage, and Tregs are well-known to express high levels of the IL-2 receptor (CD25⁺) highly in their

surface. Therefore, we expected IL-2 to be an important factor to enable contactless bystander suppression of Tregs. We confirmed the role of IL-2 during the initial co-culture of MBP-specific T effectors and MBP-specific Tregs by monitoring the phosphorylation of STAT5 in Tregs and T effectors, respectively. As shown in figure 5A, activated Ob2F3 Tregs failed to show phosphorylated Stat5 in the absence of Ob2F3 T effectors because there was no IL-2 initially produced in the culture. However, Stat5 in Tregs became phosphorylated as early as 8 hrs after co-culture with T effectors, and this was prolonged over 72 hrs (Fig. 5A). Under the same activating conditions, STAT5 phosphorylation of T effectors began within 8 hrs of stimulation with or without co-cultured Tregs. Importantly, this phosphorylation in effector T cells was rapidly curtailed in the presence of co-cultured Tregs (Fig. 5B). These data strongly suggest that there are two sequential steps in bystander suppression of specific Tregs: complete activation of Tregs by IL-2 from T effectors, followed by inhibition of the IL-2 signaling pathway of T effectors to suppress.

To further address the role of activation of Tregs with IL-2 in contactless bystander suppression, Ob2F3 T effectors and Ob2F3 Tregs were stimulated with γ -irradiated PBMCs loaded with MBP 85-99 together (Fig. 5C, **part II**) or separately (Fig. 5C, **part III**) in transwells in these presences of indicated doses of IL-2 (Fig. 5D). To determine the dose of IL-2 required to allow distant suppression, we examined IL-2 responsiveness of Treg and T effectors, respectively, by measuring Stat5 phosphorylation in serial dilutions of IL-2 (Supplemental Fig. 4). As demonstrated by others(13, 14), Tregs did not significantly suppress the proliferation of contactless bystander T effectors in the absence of IL-2 (**0 IU/ml**, Fig. 5D),. With high doses of IL-2 (**10 IU/ml**, Fig. 5D), Tregs failed to suppress either contacted or contactless T effectors. However, Tregs activated by a low dose of IL-2 remarkably suppressed the proliferation of contactless T effectors. Taken together, we concluded that IL-2 secreted from neighboring T effectors promotes the activation of single clonal engineered Tregs, enabling them to suppress contactless bystander T effectors by secreting soluble suppression factors.

Discussion

Regulatory T cells (Tregs) have been used recently for clinical therapy of a variety of adverse immune disorders. For example, Treg therapies have already been applied in clinical studies for prevention of graft *versus* host disease and type I diabetes (1, 15–17). However, polyclonal Tregs encompass many specificities and could potentially be immunosuppressive, possibly leading to viral reactivation (1). We proposed that the use of antigen-specific Tregs would be preferable for future gene therapeutic approaches for both safety and efficacy.

Expansion of specific Tregs from transgenic mice was previously reported by Anderton and colleagues (18, 19). In this report, we demonstrate that human peripheral blood CD25⁺ Tregs can be rendered “mono-specific” for MBP via transduction of TCR V genes from an MS patient’s T cell clone. These engineered Tregs suppressed the proliferation and cytokine formation of MBP-specific T effectors, even in the presence of strong inflammatory signals by TLR agonists. We also showed that these Tregs, while specific, can promote bystander suppression to proximally activated cells *in vitro* and can modulate MOG-induced EAE *in vivo*.

MBP-specific Tregs, activated with initial IL-2 secreted by neighboring T effectors, suppressed bystander T effectors with other antigen specificities via soluble suppression factors in local milieu, thus supporting their use as a potential therapy for MS even when the target CNS antigens are not precisely known. From these results, we suggest that MBP-specific single clonal Tregs completely activated with cognate MBP peptide-MHC complex and IL-2, secreted secondary suppressive factors to shut down the IL-2 signaling pathway of contactless bystander pathogenic T effectors in local milieu (Fig. 6). In a recent study, we similarly demonstrated that once Tregs are specifically activated, they can suppress T effector cells and antibody production from B cells *in vitro* and *in vivo* (5).

Tregs has been reported to produce many secreted suppressive factors to affect both contact-dependent and contact-independent suppression (e.g. TGFbeta1, IL-10, IL-35, etc.), as well as cell-surface suppressor molecules (e.g. CTLA-4, LAP, GARP, and GITR, etc.)(20–26). However, there has been no attempt to utilize therapeutically engineered human Tregs to ameliorate a practical pathogenic autoimmune disease model so far. In this study, we clearly showed that our MBP-specific Tregs mediate suppression of different antigen-specific bystander T effectors *in vitro* (Fig. 2) and *in vivo* (Fig. 3). These data demonstrate that once activated Tregs with cognate antigen-MHC complex can regulate the activation of T effectors via cell-cell contact and/or contact-independent manner without any limitation of TCR repertoire. Although contact-independent suppression is a suggested mechanism to suppress T effectors with different specificity (e.g. MOG in this study), bystander suppression effect would be restricted in local inflammatory milieu because contact-independent suppression is relatively weaker than cell-cell contact manner and their spectrum and the strength relies on the physical distance from the activated Tregs (Fig 4). There was no significant infection event due to non-specific suppression in Treg-infused EAE mice. Through figure 4 and 5, we also demonstrated successful IL-2 triggered contact-independent bystander suppression, however, the identity of the soluble suppressive factor(s) is still unknown and currently being investigated. Considering the rapid dephosphorylation of STAT5 in T effectors by co-activated Tregs (Fig. 5), the soluble suppressive factors might possibly target IL-2 downstream signaling pathway or the continued production of IL-2 in T effectors.

We have previously demonstrated that FVIII-specific TCR engineered Tregs [6], as well as single chain CAR Tregs(6), inhibit the production of FVIII-specific antibodies using an *in vitro* re-stimulation of murine FVIII-specific memory B cells. In addition, Eshhar and colleagues pioneered the use of engineered Tregs to treat a murine model of colitis (27, 28) and MacDonald et al. reported the use of CAR-engineered Tregs in a transplant model (29). As cited earlier, expansion of TCR-transgenic MBP-specific murine T cells has been used to treat EAE (19); however, the expansion of CNS-specific Tregs from a normal or patient T cell repertoire is challenging. Moreover, it is not clear whether patient's Tregs are fully functional, as suggested by the data of Baecher-Allan and colleagues (30, 31). Our approach allows for the expansion of exogenous Tregs rendered specific by transduction of a patient's TCR V regions, and the potential to overcome defects in the patient's regulatory pathways. One possible concern in the clinical translation of MBP-specific Tregs to the patients with MS is the conversion issue of the plasticity of Tregs to become T effectors in the patients *in vivo*. In the local environment of the pathogenic target tissue, this is theoretically possible,

but we showed functional stability of MBP-specific Tregs against aggressive inflammatory conditions *in vitro* (Supplemental figure 3). A few recent early phase I clinical studies with the infusion of polyclonal Tregs, in the patients with type 1 diabetes and blood cancer, did not show any serious sign of the pathogenic conversion (*e.g.* fatigue, high fever, or severe inflammations) (1, 17). Nonetheless, further clinical study in MS is needed to be investigated to address the issue.

One limitation of our approach is a xenogeneic infusion of human Tregs into immunocompetent mice. Infused human Tregs are presumably rejected over time by the host mouse immune system. This is supported by the observation of high titer of anti-human PBMC antibody in human Treg-infused mice serum at two weeks of transfer (unpublished data), implying that Ob2F3 human Tregs suppress MBP-specific mouse T effectors only temporarily during the early inflammation period. To examine the long-term effects of engineered Tregs *in vivo*, co-infusion of MBP-specific human effector T cells with Ob2F3 or control Tregs into immunodeficient mice is required. Alternatively, in the future, we plan to introduce the Ob2F3 TCR into mouse Tregs, thus allowing us to track the Tregs and determine their stability.

The clinical EAE scores and histopathologic analyses showed a therapeutic effect of Ob2F3 Tregs in the spinal cord of MOG-induced EAE mice at day 9 and day 17 (Fig. 3A and 3F), which may be due to the suppression of IFN γ production by the specific Tregs. The role of the IFN γ in MS and EAE is controversial. Although IFN γ has been considered as a prominent pro-inflammatory cytokine in MS and EAE pathogenesis (32–34), a number of subsequent animal and clinical studies have shown that IFN γ plays a protective role in MS and EAE severity by Th17 suppression (35–41). Considering the strong inhibition of IFN γ -producing Th1 cells by Ob2F3 Tregs, we predict that Ob2F3 Tregs suppress IFN γ -producing effector cells within the local milieu potentially changing the balance of Th1/Th17 prevalence. Recently Crome et al. successfully demonstrated the Th17 cell suppression by Tregs *in vitro* (42), strongly implying that Th17-driven MS pathogenesis could be controlled over the period by autologous MS-specific Tregs infusion in the patients with MS.

In summary, the observation that TCR transduced human Tregs can be functional in an immunocompetent and inflammatory environment in MOG-immunized mice also suggests that they may provide a suppressive environment, and have potential to be effective in an autologous setting in the future.

Methods

General reagents, human peripheral blood, and animals

Recombinant human IL-2 was from the NCI Biological Resources Branch Preclinical Repository (NCI, Frederick, MD). Phosphorothioate-backboned random deoxyoligonucleotide (ODNps25) (12) was synthesized using "machine mixed bases" by TriLink Biotechnology. Buffy-coat fractions (20–70 year old healthy men and women) were provided by Department of Transfusion Medicine at the National Institutes of Health (NIH, Bethesda, MD) or purchased from the American Red Cross (Rockville, MD). All procedures were approved by the Uniformed Services University of the Health Sciences Institutional

Review Board. All blood donors were provided written informed consent in accordance with the Helsinki Declaration. For the *in vitro* suppression assay under inflammatory conditions, human TLR1-9 agonist kits (10 ligands) were purchased from Invivogen. HLA-DR15 transgenic mice (Tg(HLA-DR15)#Lfug) that is DRA*0101/DRB1*1501 (HLA-DR15) expressed in MHC II-deficient (DBA/2 × C57BL/6) F1 strain were provided by Dr. Lars Fugger (Oxford [43]).

Construction and retroviral production of recombinant TCR recognizing myelin basic protein (MBP) 85-99

The nucleotide sequences for α and β chain of MBP 85-99-specific human TCR, abbreviated Ob2F3 TCR, were provided by Dr. Kai Wucherpfennig (11, 44, 45). To construct recombinant TCR for retroviral expression, the nucleotide sequences of variable (V) region and Junction in α and β chain were re-cloned with α chain constant region (TRAC) and β chain constant region 1 (TRBC1) of human reference TCR which were extracted from ImMunoGeneTics (IMGT) database and then codon-optimized for successful translation in human cells. Stoichiometric expression of both α and β chain of TCR was achieved with a 2A ribosomal skip peptide, followed by internal ribosome entry site (IRES) controlled green fluorescent protein (ZsGreen1). Retrovirus for the engineered TCR was produced by triple-transfections of plasmids: pRetroX-Ob2F3TCR-IRES-ZsGreen1, pCMV-GP, and pAmpho. Retrovirus were harvested 48–72 hrs post transfection, respectively, followed by viral particle (supernatant) concentration using Retro × concentrator (Clontech) at 4°C overnight.

Isolation and *in vitro* manipulation of TCR-transduced T effectors and Tregs

For the preparation of naive T cells ($CD4^+CD25^-CD127^+CD45RA^+$) and Tregs ($CD4^+CD25^{hi}CD127^{lo}$), peripheral blood mononuclear cells (PBMCs) were separated using Ficoll-Paque PLUS (GE Healthcare) and $CD4^+$ T cells were positively enriched with human CD4 microbeads (Miltenyi Biotech). FACS-sorted native T cells and Tregs were stored in liquid nitrogen (LN_2) until use. For retroviral expression of Ob2F3TCR and 17195TCR (5), thawed cells (1×10^6) were pre-stimulated with anti-CD3e (5 μ g/ml) and anti-CD28 (1 μ g/ml) antibodies for 48 hr in RPMI1640 culture media supplemented with 10% Fetal bovine serum (FBS), recombinant IL-2 (rIL-2, 200 IU/ml), and ODNps25 (2 μ M) (5, 12), followed by spinfection ($1500 \times g$, 2 hr, 32°C) onto retronectin (10 μ g/ml, company name)-coated plate. After 48 hr of transduction, transduced cells were expanded *in vitro* in the presence of rIL-2. To prepare pure Ob2F3TCR- or 17195TCR-transduced cells, GFP⁺ cells were FACS-sorted after 2-week-expansion and then re-stimulated with MBP 85-99 (2 μ g/ml) or FVIII 2191–2220 (1 μ g/ml) in the presence of γ -irradiated HLA-DR1/DR15 PBMCs ($2-5 \times 10^6$ /ml) and rIL-2 for next 7–14 days. For *in vitro* proliferation and suppression experiments, expanded GFP⁺ T effectors and Tregs were rested for 2 and 3 days, respectively, in rIL-2 deprivation.

Flow cytometry

For intracellular staining of Foxp3 and Helios, cells were first extracellularly stained with anti-CD4-PECy7 (RPA-T4, TONBO Biosciences) and Fixable Viability Dye eFluor780 (eBioscience) in FACS buffer, followed by fixation with 2% paraformaldehyde solution for

10 min at 37°C. After permeabilization in 0.1% Triton X-100 overnight, cells were further stained intracellularly with antibodies for Foxp3-APC (236A/E7, eBioscience) and Helios-PE (22F6, Biolegend). For staining of human TCR β chain of Ob2F3TCR, anti-human V β 2-PE (Beckman Coulter) antibody was used. For the intracellular production of interferon- γ (IFN γ) and IL-2, rested cells were re-stimulated with phorbol myristate acetate (50 ng/ml, Sigma-Aldrich) and ionomycin (1 μ g/ml, Sigma-Aldrich) in the presence of Golgi Stop (0.75 μ l/ml, eBioscience) for 4 hr at 37°C. Intracellular staining was performed with anti-IFN γ -PECy7 (Biolegend) antibody and anti-IL-2-PE (Biolegend) antibody. All FACS data were collected by BD FACSDiva software using instrument BD LSRII, and analyzed using FlowJo software (FlowJo, LLC).

***In vitro* proliferation, and antigen-specific or bystander suppression assay**

For *in vitro* proliferation assay, Ob2F3TCR-effector T cells (3×10^4) were washed twice in PBS and labeled with Cell Proliferation Dye eFluor450 (CPD450, 10 μ M) for 20 min at room temperature. Labeled cells were then co-incubated with γ -irradiated HLA-DR1/DR15 PBMCs (6×10^4) in the presence of MBP peptide (2 μ g/ml) or nonspecific OVA peptide without rIL-2 for 4 to 5 days. Proliferation was detected by flow cytometric analysis of CPD450 dilution in the effector cells. The *in vitro* suppression experiments were performed as described previously (5) using [3 H]-thymidine incorporation and CPD450 dilution assay. Briefly, rested Ob2F3TCR-effector cells (17195TCR-effector cells for bystander suppression experiment) ($2\text{--}3 \times 10^4$) and γ -irradiated HLA-DR1/DR15 PBMCs ($4\text{--}6 \times 10^4$) were mixed with Ob2F3TCR-Tregs at indicated ratios, followed by co-culture for 3 to 5 days in the presence of MBP 85-99 (2 μ g/ml) and FVIII 2191–2220 (1 μ g/ml) together or MBP 85-99 alone without the addition of rIL-2. For [3 H]-thymidine incorporation, [3 H]-thymidine (0.5 μ Ci per well) was added to the cells 18 hrs prior to harvest, and radioactivity (count-per-minute, CPM) were measured. Alternatively, a CPD450 dye dilution assay was performed by flow cytometric measurement of proliferation and absolute count of diluted cells. For absolute cell counting, 5000 CountBright Absolute Counting Beads (ThermoFisher Scientific) were added before flow cytometry, and the number of cells within gated population was determined based on acquiring 1000 beads with given flow rate.

Detection of secretory inflammatory cytokines using Cytometric Bead Array (CBA)

Procedures for the detection of cytokines secreted by effectors were according to the manufacturer's instructions. Briefly, on day 2 of co-culture with Tregs, culture supernatants (30 μ l) were transferred into the reaction tube and incubated with cytokine detection reagents (30 μ l) and capture beads (30 μ l) for 3 hrs shielded from light. Expression flow data were collected using BD FACSDiva software with BD LSRII.

***In vivo* suppression of MOG (35–55) induced EAE in HLA-DR15 mice**

EAE was actively induced in both male and female HLA-DR15 transgenic mice (age 4.5–7.5 months), which were divided equally among the treatment groups, by subcutaneous immunization on the flanks and base of tail with an emulsion containing 200 μ g of myelin oligodendrocyte glycoprotein peptide (MOG 35–55) and 400 μ g *Mycobacterium tuberculosis* H37Ra (DIFCO, Detroit, MI) in complete Freund's adjuvant (CFA). On the day of immunization (day 0) and 48 h later, mice also received 200 ng of pertussis toxin (Sigma-

Aldrich, St. Louis, MO) in 0.5 ml PBS intraperitoneally. To suppress EAE, mice were intravenously injected with 2×10^6 Ob2F3 TCR-transduced Tregs in 200 μ l PBS on day 7 following the immunization. Control groups were either injected with the same number of 17195 TCR-Tregs or with PBS. The severity of EAE was assessed daily with a 0 to 5 scoring system: 0, normal; 0.5, partially limp tail; 1, paralyzed tail; 2, hind limb weakness and loss in coordinated movement; 2.5, one hind limb paralyzed; 3, hind limbs paralyzed; 4, hind limbs paralyzed with fore limb weakness; and 5, moribund.

Detection of migration of infused hTregs and perivascular inflammatory infiltrates in EAE induced mice

Mouse brains and spinal cords were perfused with PBS/4% paraformaldehyde and processed into OCT blocks for sectioning. The frozen sections were cut for both H&E and immunofluorescence staining. H&E staining was performed by Histoserv, INC. and images were taken using a light microscope (Leica Microsystems, Buffalo Grove, IL) equipped with a digital camera (Leica DFC295), processed with LAS color v4 software. To detect infused Tregs, sections (10 μ m) were post-fixed for 2 min in cold-acetone and rinsed three times in PBS, permeabilized with 0.5% Triton-X100 in PBS for 20 min. Samples were incubated with antibody specific for human CD4-PE (RPA-T4, Tonbo Biosciences) at room temperature for overnight, followed by washing with PBS-T (0.05% Tween-20 in PBS). For confocal microscopic analyses, fixed and stained sections were mounted with Vectashield mounting medium with 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). Images were taken using a Zeiss LSM 700 Laser Scanning Microscope (Carl Zeiss, Thornwood NY) 40 $\times$ oil-immersion objective at ambient temperature and processed using ZEN 2012 software (Carl Zeiss).

Phosflow of intracellular STAT5

The cells reactivated with IL-2 for 20 min were immediately fixed in Cytotfix buffer (BD biosciences) for 10 min at 37°C. Fixed cells were washed once in chilled Phosflow Perm buffer (BD biosciences), and then permeabilized for 40 minutes in Phosflow Perm buffer at 0°C. Permeabilized cells were incubated with APC-conjugated anti-Stat5 (pY694) antibody (BD biosciences) and PE-Cy7-conjugated anti-human CD4 antibody (Tonbo Biosciences) for 1 hrs at 37°C, followed by flow cytometry analysis.

***In vitro* contactless suppression assay across APCs and in horizontal interwells**

For contactless suppression across APCs, MBP p85-99 (2 μ g/ml) and FVIII p2191–2220 (1 μ g/ml) were pre-loaded on γ -irradiated HLA-DR1/DR15 PBMCs (5×10^6) together or separately by incubating for 2 hrs at 37°C. These cells were washed with fresh media twice to remove excess peptides, and peptide-loaded PBMCs (4×10^4) were co-cultured with CPD450-labeled 17195 T effectors (4×10^4), Ob2F3 T effectors (4×10^4), and Ob2F3 Tregs (4×10^4) as indicated condition (as shown in Fig. 4A) for 4–5 days in the absence of IL-2. For measuring the suppression of 17195 T effectors, co-cultured cells were harvested and extracellularly stained with PE-Cy7-conjugated anti-human CD4 antibody, followed by absolute counting of CPD450-labeled CD4⁺ cells in flow cytometer. For interwell contactless suppression, Ob2F3 T effectors (4×10^4) were placed with MBP p85-99-loaded γ -irradiated HLA-DR1/DR15 PBMCs (8×10^4) in one well, and Ob2F3 Tregs (4×10^4) were

placed with same APCs in next well of a flat-bottom 96 well plate. Total volume of cell mixture was 200 μ l. The “transwell” condition in horizontal plane were next prepared by creating hole in a well between interwell space and well with flame-heated 18 G needle, followed by addition of 150 μ l of culture media into both the well and inter-well space to cover the holes with culture liquid. The cells were co-cultured for 4–5 days in the absence of IL-2, followed by [3 H]-thymidine incorporation.

Statistics

Statistical calculations were generated using GraphPad Prism and Microsoft Excel. Normality was confirmed using one-way ANOVA. Students' *t* test was used to determine significant differences between groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The details of statistical analyses are described in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Expanded human T regulatory cells (Tregs) transduced with an MBP-specific TCR from an MS patient can not only suppress the response of effector cells to specific peptide, but also can exert bystander suppression to effectors in the local milieu.
2. Tregs are activated by IL-2 produced by effector T cells.
3. Engineered specific human Tregs can ameliorate *MOG*-induced EAE in mice.

Figure 1. MBP-specific suppressive phenotypes and *in vitro* function of Ob2F3 Tregs

For the preparation of Ob2F3 T effectors and Ob2F3 Tregs, FACS-sorted polyclonal naïve T cells and Tregs were transduced with viral Ob2F3 TCR and then expanded for a total of 3 weeks after 2nd FACS sorting of GFP-positive cells. Detailed procedures are described in Methods. (A) To measure Foxp3 and Helios, and intracellular cytokines (IFN γ and IL-2), the cells were re-stimulated with PMA/Ionomycin for 4 hrs. in the presence of Golgi stop without IL-2. After intracellular staining, the production of Foxp3, Helios, IFN γ and IL-2 was analyzed in CD4⁺GFP⁺ populations (left panels in A). Numbers in the plot indicates the frequency of gated cells from the total. (B) Expression analysis of LAP and GARP. Treg cells were restimulated with γ -irradiated HLA DR1/DR15 PBMCs for 36 hours in the presence of IL-2 and specific peptide. Numbers in the plot indicates the frequency of gated cells from the total. (C) MBP-specific immunosuppression by Ob2F3 Tregs. The general

procedure is described in Methods. Briefly, GFP+ Ob2F3 T effectors and Tregs or effectors and Tregs transduced with a non-relevant control TCR (17195) were co-cultured in different Treg/T effector ratio with γ -irradiated HLA DR1/DR15 PBMCs and MBP 85-99 (2 μ g/ml) for 4 days without addition of IL-2. Immunosuppression was evaluated using 3H-thymidine incorporation assay. Data are presented as mean $\pm$ SD. Representative data from one of two (A and B) or four experiments (C) are shown.

Figure 2. Bystander suppressive effect of Ob2F3 Tregs

FVIII-specific TCR-transduced T effectors (17195) and Ob2F3 Tregs prepared as described in Methods. For effector T cell counting, 17195 T effectors were labeled with cell proliferation dye eFluor 450 (CPD450) before co-culture. Suppressive assay was performed by co-culture of CDP450-labeled 17195 T effectors (2×10^4) and Ob2F3 Tregs or 17195 Tregs at the indicated Treg/T effector ratio (x axis) in the presence with γ -irradiated HLA DR1/DR15 PBMCs (4×10^4). For activation of Tregs, MBP 85-99 (pMBP, 0.5 μ g/ml) or OVA p323-339 (pOVA, 1 μ g/ml), as a nonspecific control peptide, was added with FVIII p2191-2220 (pC2, 0.5 μ g/ml), which is a specific peptide to 17195 T effectors, at the indicated T effector and Treg ratios. Data are presented as mean $\pm$ SD. Representative data from one of four experiments are shown.

Figure 3. Ob2F3 Tregs ameliorate MOG₃₅₋₅₅-induced EAE in humanized HLA DR15 mice
Male and female HLA DR15 mice (4.5–7.5 months of age) were actively induced for EAE with pMOG₃₅₋₅₅/CFA/PT. On day 7 after immunization, the mice were intravenously injected with 2×10^6 of either engineered MBP-specific Ob2F3 human Tregs (Ob2F3) or control 17195 human Tregs (17195, $n = 9$). An additional control group was injected with PBS ($n = 5$). EAE symptoms were scored daily. Mean clinical score of EAE (**A**), and cumulative disease index (**B**) were shown. Data were pooled from two independent experiments with similar results and presented as mean $\pm$ SEM. Significance between two groups was analyzed by Mann-Whitney U test. * $p < 0.05$, Ob2F3 group vs. PBS group; # $p < 0.05$, 17195 group vs. PBS group.

0.05, Ob2F3 group vs. 17195 group. **(C)** Images of immunofluorescence with PE-conjugated anti-human CD4 (red) antibody in horizontal cryosections of the injected mouse cerebrum on day 9. Nuclei were counterstained with DAPI (blue). Original magnification 40× and inset was retaken with enlarged scope (right). Scale bar, 10 μm. Graph on right shows profile of hCD4 intensities in ascending order by the pixels of enlarged images measured by ZEN2012 software. **(D)** Two representative H&E stained spinal cord section of PBS, 17195 Treg and Ob2F3 Treg injected mice injected on day 9, and harvested on day 17. Arrow indicates perivascular brain region. Original magnification 20×. Scale bar, 100 μm.

Figure 4. Inter-well contactless suppression by Treg-T effector suppression complex
(A) Contactless suppression across APCs *in vitro*. Preparation of the cells and the suppression assay protocols were described in detail in Methods. For proliferation and conventional bystander suppression control, CPD450-labeled 17195 T effectors (4×10^4) co-cultured with HLA DR1/DR15 PBMCs (4×10^4) in the absence (part I) or presence (part II) of Ob2F3 Tregs in cognate peptide-loaded conditions for 4–5 days. To induce inter-APC bystander suppression, CPD450-labeled 17195 T effectors (4×10^4) and Ob2F3 Tregs (4×10^4) were co-cultured with MBP 85-99 peptide-loaded PBMCs (4×10^4) and FVIII 2191–2220 peptide-loaded PBMCs (4×10^4) together (part III) or in the presence of additional

unlabeled Ob2F3 T effectors (4×10^4) (part IV). The Y axis indicates absolute counts of CPD450⁺ T effectors by flow cytometer after co-culture. Statistical significance was analyzed by unpaired Student's *t* test. Data shown are representative from one of four experiments. **(B)** Concept of Interwell contactless suppression. Interwell space utilized as a buffering space for separate T effector and Treg culture (de-cellularized zone; DCZ). In these culture plates, T effector and Treg cultures are separated by DCZ (I). By making a hole between well and DCZ, culture media but not cells will diffuse to the DCZ (II), and eventually will be equilibrated over the cell culture space via DCZ (III). **(C)** For contactless suppression, Ob2F3 T effectors (2×10^4) were co-cultured with Ob2F3 Tregs (2×10^4) or alone (as the indicated combination in the figure) in the presence of γ -irradiated HLA DR1 PBMCs (4×10^4) with MBP 85-99 (2 μ g/ml). For performing contactless suppression assay, T effectors with PBMCs (part I), T effectors and Tregs separately with PBMCs (part II), T effectors and Treg/ complex separately with PBMCs (part III), or Treg/T effector complex with PBMCs (part IV) were co-cultured as shown in the figure. After 4 days incubation, 0.5 μ Ci 3 H-thymidiine was added at 18 hrs prior to harvest. Statistical significance was analyzed by unpaired Student's *t* test. Data shown are representative from one of three experiments.

Figure 5. Phosphorylation of Stat5 and IL-2 effect in contactless bystander T effectors and suppressive Tregs

To monitor Stat5(pY694) phosphorylation in T effectors and Tregs during the suppressive co-culture, CPD450-labelled Ob2F3 T effectors (1×10^6) were activated with DR1/DR15 PBMCs and MBP p85-99 peptide in the presence or absence of Ob2F3 Tregs for up to 72 hrs. In parallel, Ob2F3 Tregs were activated with DR1/DR15 PBMCs and MBP 85-99 peptide. The co-cultured cells were harvested at the indicated times (*x* axis), and phosphorylation of Stat5 (pY694) was examined by flow cytometry as described in Methods. Status of pStat5 in Tregs (**A**) and T effectors (**B**) were analyzed by gating GFP⁺CPD450⁻ cells and GFP⁺CPD450⁺ cells, respectively. The Y axis indicates the percent of phosphorylated Stat5-positive (pSTAT5⁺) cells among the gated cell populations. Data shown are representative from one of three independent experiments with different donors. (**C**) To demonstrate the IL-2 effect in contactless bystander suppression, Ob2F3 T effectors (3×10^4) were co-cultured with (part II) or without (part III,) Ob2F3 Tregs (3×10^4) in the presence of HLA DR1/DR15 PBMCs (6×10^4) and MBP 85-99 peptide (2 μ g/ml) on the bottom well of conventional 96 trans-well plate (Corning, NY). For contactless bystander suppression, Ob2F3 Tregs and APCs was added on the meshed top and Ob2F3 T effector and APCs was on the bottom (part III), and then co-cultured for 4–5 days in given dose of IL-2 condition (shown in **D**) The proliferation of T effectors was measured by [³H]-thymidine incorporation. Statistical significance was analyzed by unpaired Student's *t* test

(*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Data shown are representative from one of three experiments.

Figure 6. Suggested mechanism for contact-independent suppression of effector T cells specific for MOG by MBP-specific engineered Tregs

MOG or MBP-specific T cell clones are initially activated with production of IL-2 by MOG or MBP-presentation by APCs. Effector cell produced IL-2 provides a strong secondary signal during the expansion of MOG- or MBP-specific T effectors in draining lymph nodes and inflamed CNS, resulting pathogenic demyelination. With an infusion of MBP-specific single clonal Tregs, these engineered Tregs are activated upon the presentation of MBP p85-99 peptide and IL-2 secreted from T effectors with sharing of APCs or separately in a proximal distance. Activated Tregs subsequently secrete soluble suppressive factor(s) to enable the contactless bystander suppression in the local inflamed milieu, suppressing further proliferation of localized pathogenic T effectors by shutdown of phosphorylation of Stat5, a critical downstream signaling pathway of IL-2.