# Genetically modified hematopoietic stem/progenitor cells that produce IL-10–secreting regulatory T cells

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**Random amino acid copolymers used in the treatment of multiple sclerosis in man or experimental autoimmune encephalomyelitis (EAE) in mice [poly(Y,E,A,K)$_n$, known as Copaxone, and poly(Y,F,A,K)$_n$] function at least in part by generation of IL-10–secreting regulatory T cells that mediate bystander immunosuppression. The mechanism through which these copolymers induce Tregs is unknown. To investigate this question, four previously described Vα3.2 Vβ14 T cell receptor (TCR) cDNAs, the dominant clonotype generated in splenocytes after immunization of SJL mice, that differed only in their CDR3 sequences were utilized to generate retrogenic mice. The high-level production of IL-10 as well as IL-5 and small amounts of the related cytokines IL-4 and IL-13 by CD4$^+$ T cells isolated from the splenocytes of these mice strongly suggests that the TCR itself encodes information for specific cytokine secretion. The proliferation and production of IL-10 by these Tregs was costimulated by activation of glucocorticoid-induced TNF receptor (GITR) (expressed at high levels by these cells) through its ligand GITRL. A mechanism for generation of cells with this specificity is proposed. Moreover, retrogenic mice expressing these Tregs were protected from induction of EAE by the appropriate autoantigen.**

multiple sclerosis | immunosuppression | retrovirus | cytokines

The random amino acid copolymers poly(Y,F,A,K)$_n$ (called YFAK), and poly(Y,E,A,K)n [YEAK, Copaxone, a drug widely used to treat multiple sclerosis (MS)] induce the differentiation of regulatory T cells that secrete IL-10 (1). This cytokine mediates bystander immunosuppression and leads to amelioration of experimental autoimmune encephalomyelitis (EAE), the murine model of the human disease MS. YFAK-specific T cell lines have been generated from SJL mice treated with YFAK, and the nature of their T cell receptors has been investigated (2). Interestingly, these regulatory T cells preferentially used Vα3.2Vβ14, and, to only a slightly lesser extent, Vβ4 with more variable Vα segments. Engagement of their TCR by YFAK presented by I-A$^s$ in irradiated SJL splenocytes led to the production of high levels of IL-10. These Treg cell lines are Foxp3$^-$ and may be closely related to the Foxp3$^-$ Tregs called Tr1 cells that have been extensively studied (3). We hypothesized, for reasons described below, that some peptide within the sequence of the TCR may be responsible for the expansion of a small pool of precursors and/or for the conversion of naïve T cells to selective IL-10 secretion. To test this hypothesis, retrogenic mice (4–6) expressing large numbers of regulatory T cells were generated using four TCR Vα3.2 and TCR Vβ14 cDNA clone pairs obtained from spleens of mice that had been treated with YFAK (2). These retrogenic mice expressed large numbers of CD4$^+$ Vβ14$^+$ regulatory T cells in spleen, lymph node, and thymus, which secreted IL-10 and related cytokines upon stimulation with YFAK and, moreover, were protected from induction of EAE. These experiments strong suggest that the TCR itself encodes the information for specific cytokine secretion in these IL-10–secreting Tregs, a previously unrecognized function for a TCR, and suggest a possible therapy for aggressive forms of MS.

## Results

**Generation of Retrogenic HSC/HPC.** Transduction of murine hematopoietic stem/progenitor cells (HSC/HPC) with the TCR of IL-10–secreting regulatory T cells was performed. HSC/HPC are highly enriched in the c-kit–positive, Sca-1–positive, lineage marker-negative (KSL) fraction of mouse bone marrow cells. The expression level of CD34 antigen distinguishes hematopoietic stem cells (HSC) from their progeny: long-term multilineage repopulating cells. Common lymphoid progenitors (CLP), which are long-term repopulating cells, are found more frequently in the CD34$^-$ KSL fraction of the mouse bone marrow, whereas short-term repopulating cells are present in the CD34$^+$ KSL fraction. Moreover, the proportion of cells that show in vivo T cell differentiation potential among CD34$^-$ KSL cells is significantly larger than that among CD34$^+$ KSL cells. Bone marrow was extracted from the femur and tibia of SJL mice and hematopoietic progenitor cells (HPC), containing both HSC and CLP, were prepared using a standard procedure that yielded both CD34$^+$ and CD34$^-$ KSL cells (7). To obtain cells expressing our TCR of interest, these HSC/HPC were infected with retroviruses prepared using the murine retroviral vector pMIG into which had been cloned the TCR cDNA of each of the four TCR Vα3.2Vβ14 pairs marked in figure 4B of ref. 2, linked by the 2A peptide, as well as GFP (6). All had different, although related,

### Significance

Amino acid copolymers that are used in the treatment of experimental autoimmune encephalomyelitis (EAE) in mice or multiple sclerosis (MS) in humans result in the induction of immunosuppressive IL-10–secreting regulatory T cells. The TCRs of these T cells have previously been isolated and sequenced. Introduction of these TCR into a retroviral vector was used to transduce murine hematopoietic stem/progenitor cells (HSC/HPC). These genetically modified HSC/HPC were then transplanted to create retrogenic mice. Splenocytes or T cells of these retrogenic mice produced high levels of IL-10 on appropriate stimulation, and strongly suggests that the TCR itself encodes information for IL-10 secretion. Moreover, these retrogenic mice were resistant to induction of EAE, suggesting an approach to therapy for progressive MS using genetically modified HSC/HPC for transplantation.

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CDR3 sequences. Two different approaches for retrovirus infection were tested, and the procedure that yielded the best infection efficiency, evaluated by GFP expression with flow cytometry due to the presence of IRES-GFP sequences in the retroviral vector, was employed.

**Production and Analysis of Retrogenic Mice Expressing Tregs.** Next, these transduced HSC/HPC were used for in vivo development of IL-10–secreting regulatory T cells in SJL mice. Retrogenic SJL mice were successfully made using two of the TCR Vα3.2 Vβ14 pairs from IL-10–secreting regulatory T cells induced by the amino acid copolymer YFAK that differed only in the CDR3 sequences (figure 1 in ref. 2). These pairs accounted for about 30% of the TCR while Vβ4 TCR pairs that had a more variable TCRα usage accounted for about 24%. In this in vivo experiment, we could be certain that T cells would be selected in the thymus on I-$A^s$. HSC/HPC were prepared from the bone marrow of SJL mice as described above and transduced with the retrovirus vector encoding TCR1 or TCR2, two of the TCR Vα3.2 Vβ14 pairs (TCR αβ pairs 1 and 7 in figure 4B of ref. 2), and with an "empty" vector control. The transduction frequencies of HSC/HPC, as measured by GFP expression from the vectors, were in the range of 30–60%. Then, $1 \times 10^6$ transduced HSC/HPC were injected i.v. into 4-wk-old SJL mice that in this initial experiment had been irradiated with 950 rads to ablate the bone marrow (referred to as retrogenic mice; refs. 4 and 6). Tail bleeds and FACS to analyze reconstitution 5–6 wk after transfer indicated that 17% of $CD4^+$ peripheral blood mononuclear cells expressed GFP in the case of TCR1 and 33% in the case of TCR2 (Fig. 1*A*, *Upper*). Furthermore, in the case of TCR1 and TCR2, 85% and 69% of the $GFP^+$ cells were $CD4^+$ (Fig. 1*A*, *Right*), and 94% and 97% of the $CD4^+$ $GFP^+$ cells, respectively, were Vβ14 (Fig. 1*B*). The preferential differentiation of HSCs to become $CD4^+$ $Vβ14^+$ cells was specific to the $GFP^+$ YFAK-specific TCR population. The $GFP^+$ population from both TCR1 and TCR2 retrogenic mice contained more $CD4^+$ cells compared with vector control mice, whereas the $GFP^-$ population in all three cohorts had a phenotype similar to the $GFP^+$ vector control (Fig. 2). Therefore, the skewed $CD4^+$ $Vβ14^+$ population was not observed in cells that were not transduced with TCR retrovirus and was specific to the expression of the YFAK-specific T cell receptor.

Retrogenic mice were further analyzed by harvesting various tissues of the lymphatic system (Fig. 3). Similar to peripheral mononuclear cells, $GFP^+$ cells from the spleen, lymph nodes, and thymus of TCR2 retrogenic mice were predominantly $CD4^+$ $Vβ14^+$. This phenotype was not seen in any of the tissues from vector control mice. Thus, bone marrow transplantation of donor YFAK-specific TCR retrovirus transduced HSCs led to successful engraftment and subsequent expression in all lymphatic tissues, with the preferential differentiation of HSCs into $CD4^+$ T cells that express Vβ14.

**Massive IL-10 Secretion by T Cells from Retrogenic Mice.** Next, splenocytes were isolated from a TCR1 and a TCR2 mouse, as well as from a vector control mouse, the yields being $2 \times 10^8$ cells from both vector and TCR1 mouse and $6 \times 10^7$ cells from the TCR2 mouse that was smaller in size. T cells enriched with the MACS Pan TCR isolation kit were sorted for $CD4^+$ $GFP^+$ cells, yielding $10^5$ to $10^6$ $GFP^+$ $CD4^+$ T cells. After stimulation with 50 μg/mL YFAK, the amount of IL-10 produced was astonishing (i.e., ~10,000 pg/mL per $10^5$ cells in both cases) while negligible amounts were produced by vector control cells (Fig. 4*A*). The amount was so large that IL-10 precipitated in the culture supernatant. The amount of IL-10 produced under similar circumstances by splenocytes from mice treated with YFAK that are resistant to EAE was in the range of 1,000 pg/mL per $10^5$ cells (1). This experiment has been repeated and extended more than 10 times using a modified procedure in which two doses of 450 rad separated by 2 h are given and which resulted in a more stable, conditioned mouse. Secretion of antiinflammatory cytokines IL-10, IL-5, IL-13, IL-4, and of the proinflammatory cytokines IL-1β and TNFα as controls was measured (Fig. 4 *B* and *C*). The amount of IL-10 secreted using the modified procedure was 1,000–3,500 pg/mL per $10^5$ cells, still a very high level. The amount of IL-5 was similar. The amount of IL-13 in one experiment was 900 pg/mL per $10^5$ cells and of IL-4 about 500 pg/mL per $10^5$ cells.

To show that YFAK-specific T cells have a regulatory function, an in vitro T cell suppression assay was performed (Fig. 5). These YFAK-specific Tregs were cocultured with conventional peripheral T cells (Tconv) that were labeled with CFSE dye and analyzed for dilution of dye signal as a readout for proliferation

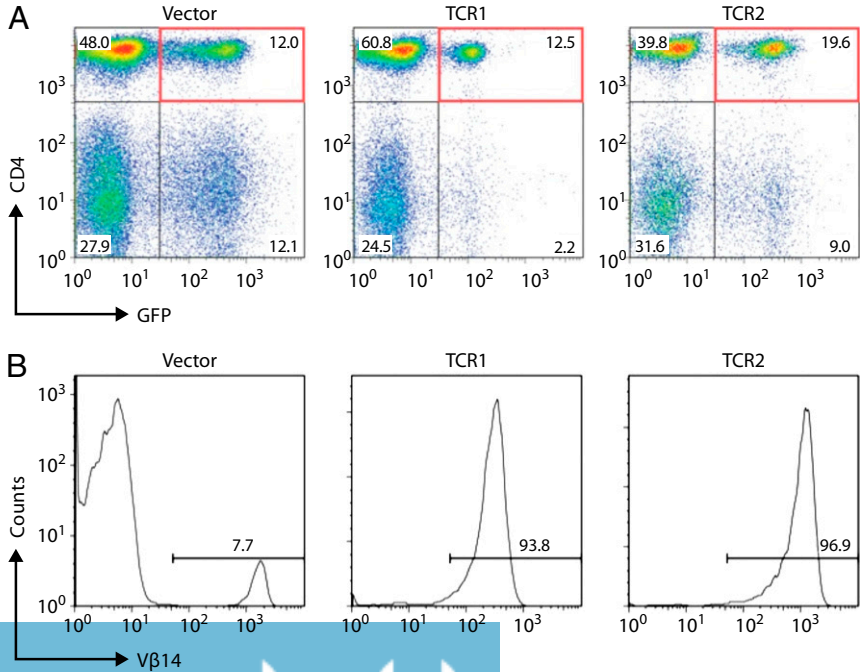


**Fig. 1.** $GFP^+$ $CD4^+$ peripheral blood mononuclear cells from TCR retrogenic mice are nearly all $Vβ14^+$. Peripheral blood mononuclear cells were isolated from tail vein, stained with CD4 and Vβ14 antibodies, and analyzed by flow cytometry. (*A*) CD4 and GFP expression in peripheral blood mononuclear cells. The red box is the gate for *B*. (*B*) TCR Vβ14 expression in $GFP^+$ $CD4^+$ gated cells. This experiment is representative of at least three independent experiments.

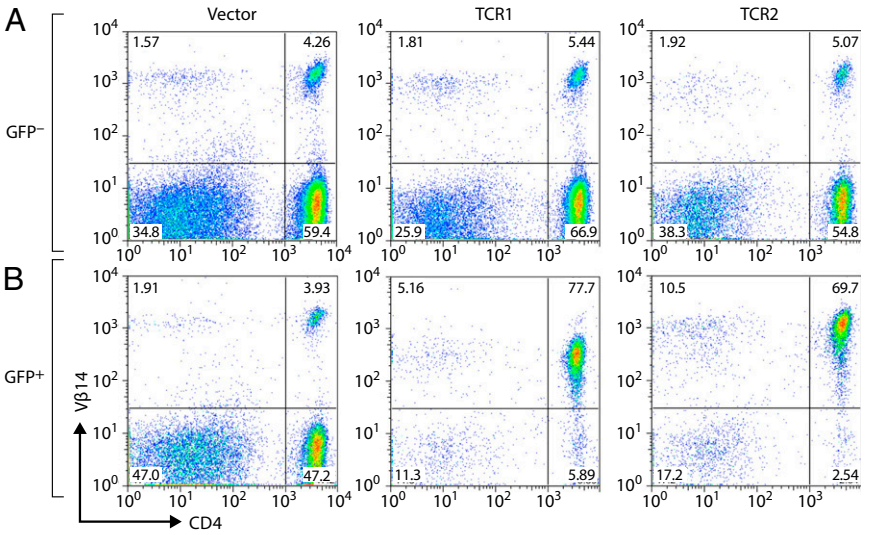


**Fig. 2.** GFP⁻ and GFP⁺ peripheral blood mononuclear cells from TCR retrogenic mice have different phenotypes. Peripheral blood mononuclear cells were isolated from tail vein, stained with CD4 and Vβ14 antibodies, and analyzed by flow cytometry. Cells were gated on either GFP negative (*A*) or GFP positive (*B*) populations, and dot plots generated were based on CD4 and Vβ14 staining. This experiment is representative of at least three independent experiments.

on addition of anti-CD3 and anti-CD28. Tconv cells alone proliferated well in culture, and their proliferation was inhibited in a dose-dependent manner with the addition of increasing numbers of Tregs. Presumably, this Tconv cell suppression was due to cell contact-independent inhibition through the effects of the antiinflammatory cytokines IL-10 and IL-5, produced by the Tregs. To rule out the possibility of contact-dependent inhibition, Treg cells were cocultured with Tconv cells in a transwell system where the two different cell types are separated by a semipermeable membrane, and Tconv cells were similarly inhibited.

**GITRL as Costimulatory Ligand.** Finally, the effect of glucocorticoid-induced TNF receptor ligand (GITRL), a possible costimulatory molecule, on proliferation and IL-10 secretion was examined. Importantly, the T cell line previously studied expressed a high level of GITR [glucocorticoid-induced TNF receptor superfamily member 18 (TNFRSF18)] (1, 8). Its ligand, GITRL [TNF superfamily member 18 (TNFSF18)] (8, 9), has been reported to exist on microglia and on plasmacytoid dendritic cells (10–12) and to costimulate the secretion of large amounts of IL-10 (2). In addition, GITR ligation has been found to promote T cell proliferation.

Can GITRL activate these Tregs? The GITR receptor is expressed predominantly on regulatory T cells and has been identified to be important in Treg function. The murine GITRL dimer has been produced for X-ray structure determination (13). This material was able to induce the proliferation of these Tregs but did not itself activate IL-10 production. YFAK stimulation was required for increased IL-10 production (Fig. 6). The costimulation of YFAK and GITRL led to robust IL-10 production compared with YFAK alone. Thus, GITR activation and stimulation of the TCR specific for YFAK are both required for maximal IL-10 production. The nature of an endogenous ligand for this TCR remains to be studied.

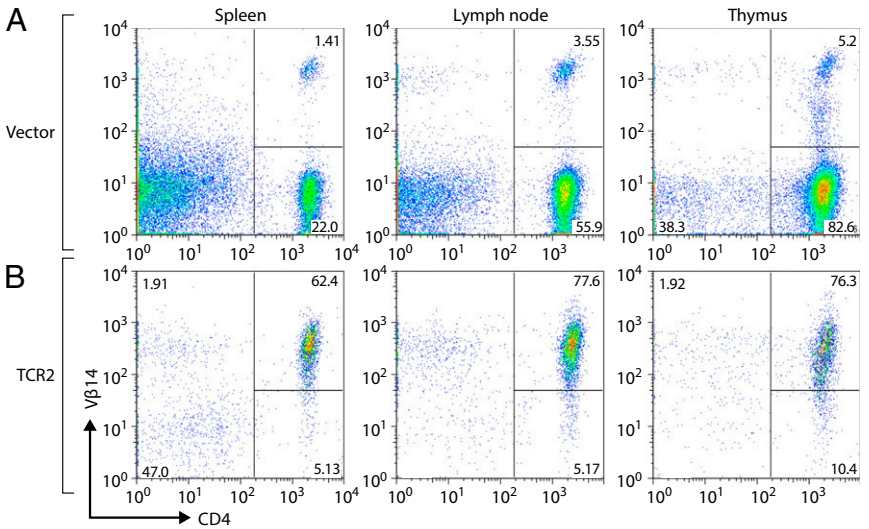


**Fig. 3.** GFP⁺ TCR2 retrogenic cells are mainly CD4⁺ Vβ14⁺ in all organs of the lymphatic system. Immune cells were harvested from various lymphatic organs of retrogenic vector (*A*) or TCR2 (*B*) mice, stained with CD4 and Vβ14 antibodies, and analyzed by FACS. Cells were gated on GFP positive population and dot plots generated based on CD4 and Vβ14 staining. This experiment is representative of at least three independent experiments.

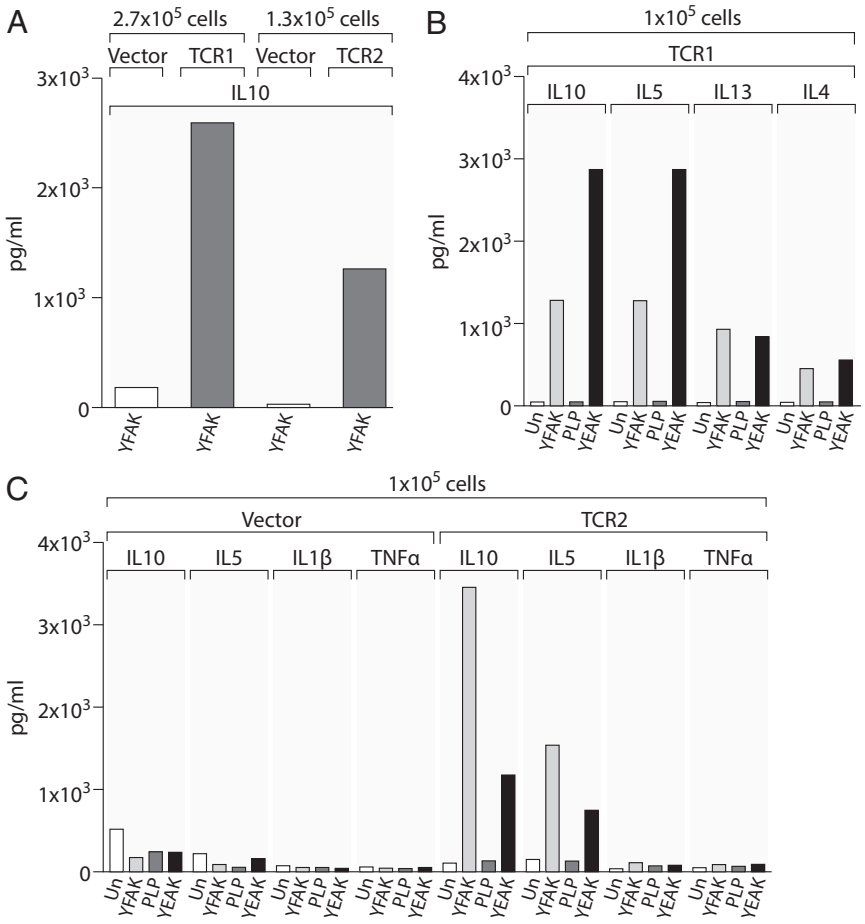


**Fig. 4.** Cytokine secretion profile from three different experiments. (*A*) IL-10 ELISA of supernatant from YFAK-stimulated CD4$^+$ GFP$^+$ TCR1 or TCR2 expressing FACS sorted splenic T cells from retrogenic mice. (*B*) Bioplex analysis using splenocytes from TCR1 expressing retrogenic mice and analyzing secretion of IL-10, IL-5, IL-13, and IL-4. (*C*) Bioplex analysis of antiinflammatory cytokines IL-10 and IL-5 and proinflammatory cytokines IL-1β and TNFα from supernatant of YFAK-treated CD4$^+$ GFP$^+$ purified TCR2 expressing FACS sorted splenic T cells. This experiment is representative of at least three independent experiments.

These data suggest that two signals are required for maximum proliferation and secretion of IL-10 from these Tregs, just as two signals are required for other T cell functions. The expression of GITRL on CNS microglia (10) may have an important effect on the course of therapy of EAE and MS with amino acid copolymers. Moreover, localized secretion within the CNS could account for the lack of generalized immunosuppression during treatment.

**Protection from EAE in Retrogenic Mice.** SJL mice that have been transplanted at 4 wk of age with HSC transduced with TCR2 Vβ14 Vα3.2 and allowed to recover for 8 wk were employed. Immunization was carried out at 12 wk with 100 μg of PLP139–151. None of the animals whose HSC had been transduced with TCR2 Vβ14 Vα3.2 developed full-blown EAE while all of the control mice whose transplanted HSC had been transduced with the same vector without the TCR were either moribund or dead at the termination of the experiment at 41 d (Fig. 7). Clearly, the transduced TCR protected these mice from induction of EAE.

## Discussion

These experiments establish that genetically modified murine HSC/HPC have been generated that lead to production of IL-10–secreting regulatory T cells after transplantation and can protect mice from induction of EAE. These techniques may be adaptable to human studies in patients with aggressive MS and, possibly, in other autoimmune diseases that may have a defect in regulatory T cells. Autologous HSC/HPC transplantation is being used to manage aggressive cases of MS with the best results obtained in aggressive relapsing remitting MS (14–20). The rationale for this procedure is that myeloablative or nonmyeloablative conditioning regimens used in preparation for transplantation will remove autoreactive T cells that induce disease while the new immune system generated with HSC/HPC will be free of these autoreactive cells. Moreover, a defect in MS patients in regulatory

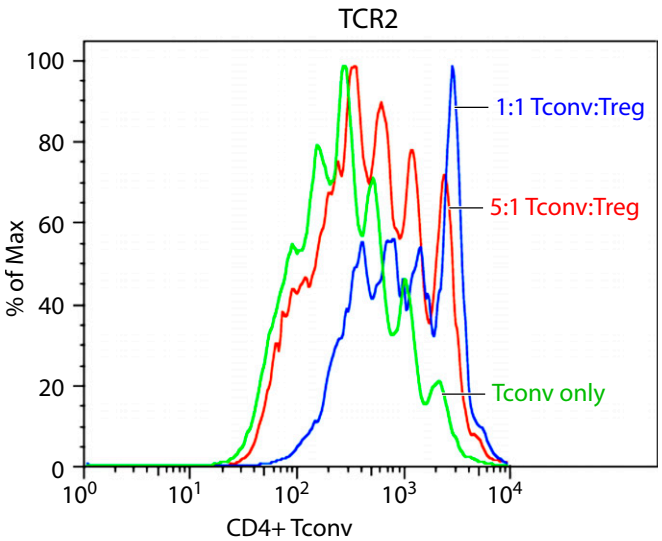


**Fig. 5.** YFAK-specific regulatory T cells can suppress conventional T cells. Different ratios of Tregs are incubated with Tconv cells and cultured for 2 d. Cells were stimulated with anti-CD3/anti-CD28. Tconv cells are labeled with CFSE to measure proliferation. This experiment is representative of at least three independent experiments.

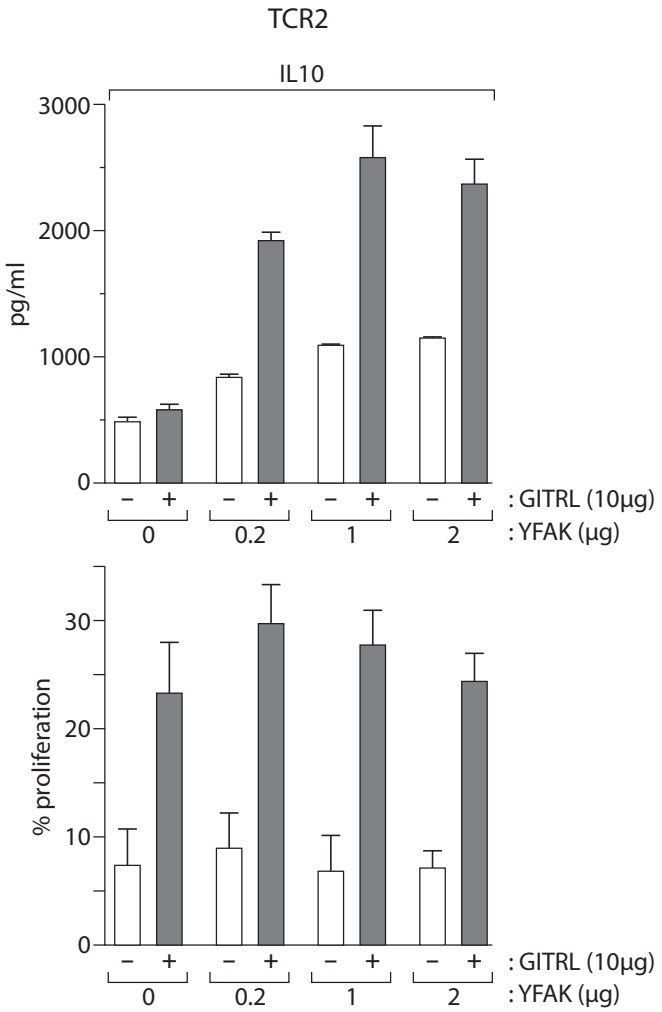


**Fig. 6.** GITRL increases proliferation of Treg cells while YFAK is required for activation of IL-10 production. YFAK-specific Treg cells were unstimulated, stimulated with GITRL or YFAK alone, or costimulated with both GITRL and YFAK, and analyzed for IL-10 production and Tconv proliferation. The *P* values for no added GITRL versus added GITRL ligand in the presence of YFAK are $P < 0.0088$ for IL-10 secretion and $P < 0.0007$ for proliferation. This experiment is representative of at least three independent experiments.

T cells has been identified (21). Additionally, the data suggest that some aspect of the TCR of these IL-10–secreting Tregs encodes the information for specific cytokine secretion. We hypothesize that it is a peptide derived from the TCR by its proteolysis at the double positive thymocyte stage. The peptide could function either by selection and expansion of IL-10–secreting Tregs or by induction of a minority T cell population with the appropriate specificity. An alternative interpretation is that selective deletion of the PLP139–151 reactive T cells could account for the data in Fig. 7. This explanation seems very unlikely in view of the vector control result and the similarity of the GFP$^-$ CD4$^+$ subsets in the control and experimental populations in Fig. 2.

The postulate that a peptide derived from the TCR may be responsible for expansion of a small precursor pool or for induction of IL-10–secreting Tregs is based on the observation that the level of cell surface expression of the TCR at the double positive (CD4$^+$ CD8$^+$) stage of T cell development is very low, perhaps 10–20% of that at the single positive stage (22, 23). This reduced level has been ascribed to proteolysis, mediated by the ubiquitination of the CD3 subunits followed by endocytosis of the TCR complex and lysosomal degradation (24). Either deletion of the E3 ubiquitin ligase c-CBL, which targets the TCR for degradation, or inhibition of the dynamin motor required for endocytosis restored the TCR level of double positive thymocytes to that found in single positive T cells. This degradative system may be the same as that employed to degrade pre-TCRs at the double negative 4 thymocyte stage (25, 26). Moreover, immunization with some peptides derived from the TCR resulted in protection from EAE and the induction of Tregs (27–30). Most interestingly, in one case, immunization with a peptide derived from a TCRβ framework region of a pathogenic Vβ8.2 clone from B10.PL mice was shown to induce Tregs, and, remarkably, preferential usage of Vα3.2 and Vβ14 was also observed (30). It has been suggested that many TCRs, regardless of their function or cytokine secretion profile, may contain sequences that induce self-regulation through the generation of Tregs (31). In addition, the J5 peptide that was synthesized based on the binding motif of Copxaone to HLA-DR2 (32), a peptide derived from a helminth antigen (33, 34), and, importantly, the peptide from Vβ8.2 can all protect against EAE and the former two have been shown to induce T cells that secrete IL-10. However, the regulatory T cells generated have never been fully characterized. In still another example utilizing H-2$^u$ mice, some feature of the TCR was postulated to be important in inducing tolerance to a myelin basic protein peptide (35). Most recently, somatic cell nuclear transfer (SCNT) from T cells that secrete IFN-γ into embryonic stem (ES) cells that reprogram the transferred nuclei and remove epigenetic marks was carried out (36). These transnuclear ES cells were then used to generate mice that developed T cells that secreted IFN-γ. This experiment suggests that the TCR genes of the T cells used for SCNT may provide the information for IFN-γ secretion, although it remains possible that all epigenetic marks were not reprogrammed in the ES cell. The present experiment utilizes naked TCRs and obviates this critique. Moreover, it extends the observation to a second family of cytokines, the IL-10 family, and suggests the possibility that information for synthesis and secretion of additional cytokines may be encoded within the TCR. These publications led to the hypothesis that some physical and/or chemical feature of the TCR itself,

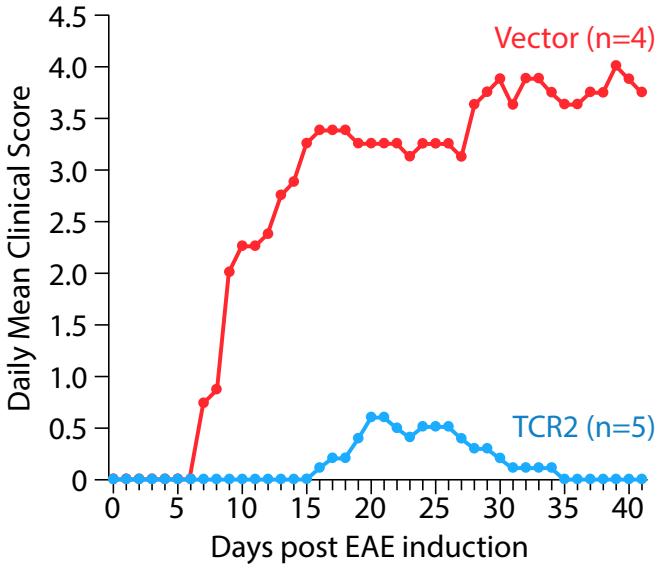


**Fig. 7.** SJL retrogenic mice that had been transplanted with HSC transduced with TCR Vβ14 Vα3.2 are protected from EAE. Vector control retrogenic mice and TCR2 retrogenic mice were injected s.c. with 100 μg of PLP139-151 emulsified in CFA at day 0 to induce EAE when the retrogenic mice were 16 wk old. Mice were scored daily, and the mean score for four vector retrogenic mice and five TCR2 retrogenic mice were plotted. The experiment was terminated after 41 d and had a *P* value <0.0001. The average peak scores ± SEM were 4 ± 0.58 and 0.6 ± 0.4 for vector and TCR2, respectively. This experiment is representative of at least three independent experiments.

presumably a specific peptide sequence, is important in generating regulatory T cells that have a specific cytokine secretion profile and, therefore, T cell phenotype.

Whether the phenomenon can be extended to the subdominant Vβ4 TCR bearing T cells found in the cell line induced by YFAK and whether it can be extended to the TCR of T cells secreting other cytokines remains for further study. Increasing the number of IL-10–secreting regulatory T cells in aggressive demyelinating diseases including the aggressive form of EAE used here, by transplantation of HSC/HPC genetically modified by transduction of an appropriate T cell receptor, may either prevent induction of the disease or effect significant amelioration (Fig. 7). Preliminary data suggest that the innate immune system is involved in the phenomenon. Myeloid cells have been reported to be involved in the generation of IL-10–secreting T cells induced by amino acid copolymers (37–39). Microglia from brains of wild-type and retrogenic mice were isolated and were found to markedly up-regulate expression of GITRL (*SI Appendix*, Fig. S1). Moreover, splenocytes from retrogenic mice up-regulated the expression of TLR2 (*SI Appendix*, Fig. S2). Whether these two phenomena are related to each other remains to be shown, but their occurrence indicates that the innate immune system is involved in the generation of IL-10–secreting T cells induced by these retrogenic TCR.

## Materials and Methods

**Mice.** SJL/J female mice (3 wk old) were purchased from Jackson Laboratory and maintained according to the Guidelines of the Committee on Animal Care of Harvard University and the Committee on Care and Use of Laboratory Animal Resources, National Research Council.

**Retrovirus Transduction.** Vector control or YFAK-specific TCRαβ retrovirus was generated, as described (2, 6). Bone marrow was isolated from donor SJL/J mice and purified for lineage negative cells by MACS (Miltenyi Biotec) according to manufacturer's protocol and grown for 2 d in tissue culture with recombinant IL-3, IL-6, and SCF. Then, donor cells were transduced with retrovirus on retronectin-coated tissue culture plates (Takara Bio) for an additional 2 d, essentially as described (5, 6).

**Bone Marrow Transplantation To Produce Retrogenic Mice.** Recipient SJL/J mice at 4 wk of age were irradiated in a cesium irradiator with 950 rads (or with two doses of 450 rads separated by 2 h) 1 d before transplantation. Retrovirus-transduced bone marrow cells were retroorbitally injected into irradiated recipient mice. Mice were checked daily and given access to sulfatrim-antibiotic water during the course of reconstitution. Hematopoetic cell reconstitution was confirmed about 5 wk after transfer.

Additional information on assays is found in *SI Appendix, Materials and Methods*.

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