Published in final edited form as:
J Immunol. 2009 January 15; 182(2): 969–979.

Lymphopenia-induced Proliferation is a Potent Activator for CD4+ T Cell Mediated Autoimmune Disease in the Retina[1]

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Abstract

To study retinal immunity in a defined system, a $CD4^+$ T cell receptor (TCR) transgenic (Tg) mouse line (βgalTCR) specific for β-galactosidase (βgal) was created and used with Tg mice that expressed βgal in retinal photoreceptor cells (arrβgal mice). Adoptive transfer of resting βgalTCR T cells, whether naive or antigen-experienced, into arrβgal mice did not induce retinal autoimmune disease (experimental autoimmune uveoretinitis, EAU), and gave no evidence of antigen recognition. Generation of βgalTCR T cells in arrβgal mice by use of bone marrow grafts, or double Tg mice, also gave no retinal disease or signs of antigen recognition. Arrβgal mice were also resistant to EAU induction by adoptive transfer of in vitro activated βgalTCR T cells, even though the T cells were pathogenic if the βgal was expressed elsewhere. In vitro manipulations to increase T cell pathogenicity prior to transfer did not result in EAU. The only strategy that induced a high frequency of severe EAU was transfer of naive, CD25-depleted, βgalTCR T cells into lymphopenic arrβgal recipients, implicating regulatory T cells (Tregs) in the T cell inoculum, and in the recipients, in the resistance to EAU. Surprisingly, activation of the CD25-depleted βgalTCR T cells prior to transfer into the lymphopenic recipients reduced EAU. Together, the results suggest that endogenous regulatory mechanisms, as well as peripheral induction of Tregs, play a role in the protection from EAU.

Keywords

Autoimmunity; Tolerance/Suppression; Neuroimmunology

Introduction

T cell recognition of central nervous system (CNS)[3] antigens (Ag) induces both tolerance that reinforces CNS immune privilege, and immunopathogenesis, when immune privilege fails to protect from autoimmune disease. The mechanisms that control the outcome of T cell recognition of CNS Ags form the basis for maintenance of peripheral tolerance in CNS tissues (1). The retina is a distinct subset of CNS tissue with an extraordinary concentration of tissue-specific proteins for visual transduction, the absence of meninges, and a lack of lymphatic drainage, which distinguish it from brain and spinal cord (2,3).

Retinal immune privilege is maintained by several activities. *Aire*-related mechanisms are important, as *aire*-driven expression of retinal interphotoreceptor retinoid-binding protein

[1]This study was supported by NIH grants EY011542 and EY016376; Research to Prevent Blindness; The Anna Heilmaier Foundation, St. Paul, Minnesota; and the Minnesota Lions and Lionesses Clubs.

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(IRBP) affected pathogenesis in murine experimental autoimmune uveoretinitis (EAU) (4,5). A positive correlation between mRNA transcripts of photoreceptor cell Ags in the thymus and resistance to EAU has been reported for rodents (6,7). Negative thymic selection of T cells specific for retinal Ag has been observed (8-10), as has positive selection of retinal-protective regulatory T cells (Tregs) (11). Induction of EAU was most potent when heterologous Ags or non-immunodominant epitopes of retinal Ags were used for immunizations (12), suggesting that endogenous photoreceptor cell Ags induced partial self-tolerance. Immunological ignorance of retinal Ags has been suggested from several studies (13,14), and it has been proposed that the lack of retinal self-Ag in draining lymph nodes (LN) can compromise the generation of peripheral tolerance against autoreactive T cells, making the retina more vulnerable to autoimmune disease (10). This concept is supported by studies testing the consequences of peripheral expression of retinal Ag via retroviral transduction of bone marrow grafts (15,16), by extraocular transgenic (Tg) expression using a class II MHC promoter or collagen promoter (13,17), and by extraocular DNA vaccination (18). These studies showed that even exceptionally low levels of extra-retinal Ag expression yielded animals whose susceptibility to EAU was lost, while other measures of the immune response were little changed.

Given the breadth of activities working to maintain immune privilege, T cells from T cell receptor transgenic (TCR-Tg) mice that recognize either endogenous or neo-self Ags will be crucial in advancing our understanding of the multifaceted processes of self-tolerance and autoimmunity. Of particular relevance are TCR-Tg mice specific to endogenous or Tg Ags expressed within the retina associated with EAU (9,10). Using mice that express hen egg lysozyme (HEL) on retina-specific promoters, in conjunction with HEL-specific 3A9 TCR-Tg T cells, a high incidence of severe, spontaneous EAU was found in the double Tg mice, despite extensive thymic deletion (9,10). To further understand the mechanisms and conditions of T cell recognition of retinal Ags leading to either tolerance or immunopathology, we created

[3]Abbreviations:

CNS central nervous system

PC photoreceptor cells

Tg transgenic

βgal *Escherichia coli* beta-galactosidase

arrβgal photoreceptor expression of βgal

GFAPβgal astrocyte expression of βgal

βgalTCR βgal-specific TCR Tg T cells

Treg regulatory T cell

EAU experimental autoimmune uveoretinitis

EAE experimental autoimmune encephalomyelitis

HEL hen egg lysozyme

TCR-Tg mice (βgalTCR mice) using the TCR from our $CD4^+$, βgal-specific, 3E9 T cell clone (19) for use in experiments with Tg mice that express *Escherichia coli* β-galactosidase (βgal) as a neo-self Ag specifically in photoreceptor cells (arrβgal mice). Unlike the HEL/3A9 model, we found the retina to be highly resistant to disease mediated by the βgalTCR T cells even in arrβgal × βgalTCR double Tg mice, but susceptible if the βgal was expressed in brain astrocytes. The only potent enhancer of retinal photoreceptor autoimmune disease was lymphopenia-induced proliferation (LIP) induced by adoptive transfer of CD25-depleted, naive βgalTCR T cells into arrβgal mice on the $Rag^{(-/-)}$ background. Tregs present in, or generated from, the T cell inoculum contributed to the resistance to EAU in arrβgal mice.

Materials and Methods

Generation and analysis of βgalTCR transgenic mice

Generation of the $CD4^+$ 3E9 T cell clone specific for the βgal peptide (YVVDEANIETHGMV) has been described (19). Founder mice carrying transgenes for 3E9 TCR α and β chains were generated (unpublished data) and crossed to normal B10.A mice. The transgene positive F_1 mice were then crossed to generate stable 3E9-α or 3E9-β Tg mouse lines. Functional 3E9-TCR Tg mice (βgalTCR mice) were generated by crossing the 3E9-α and 3E9-β Tg mice. F_1 mice were analyzed by PCR for both the α and β transgenes. Mice positive for both transgenes were assayed for proliferative responses in peripheral blood mononuclear cells (PBMC) to either βgal peptide or whole βgal protein. Mice whose PBMCs responded to βgal Ag were crossed to propagate the βgalTCR-Tg mouse line. All subsequent βgalTCR-Tg mice were assayed for the ability of PBMCs to respond in vitro to βgal Ag prior to any other analysis.

Other mice

The βgal-expressing arrβgal (previously named hi-arr-βgal), GFAPβgal, and ROSA26 Tg mice on the B10.A background have been described in detail elsewhere (13,19-21). βgal expression in rod photoreceptor cells of arrβgal mice mimics expression of endogenous arrestin (22,23), producing 150 ng βgal/retina and <0.5 ng/pineal gland. Rare, unidentified βgal-positive brain cells were seen. No other sites of expression were found. GFAPβgal mice express βgal in astrocytes of brain (175 ng/brain), retina, and optic nerve, based on activity of the GFAP promoter (24). Expression was not found in thymus of arrβgal or GFAPβgal mice. Expression of βgal in adult ROSA26 mice was low, but present in many tissues, including the thymus. Mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and University of Minnesota animal use and care guidelines. Mice were housed under specific pathogen-free (SPF) conditions on lactose-free chow.

In-vitro T cell, PBMC, and cytokine analysis

Spleen or LN cell suspensions were filtered through a 70 μm cell strainer and the red blood cells (RBC) were lysed in 0.17 M NH_4Cl. The cells were then washed 2X in phosphate-buffered saline (PBS), and resuspended in RPMI-1640 supplemented with 10 % fetal calf serum (FCS). PBMCs were prepared from heparinized blood. Routine in vitro Ag stimulation was performed with 1μM βgal peptide using βgalTCR splenocytes or purified T cells plus irradiated (3000 R) B10.A splenocytes, as Ag presenting cells (APC), at an APC/T cell ratio of 10:1. Proliferation and cytokine assays were performed in 96-well plates using 5×10^5 βgalTCR splenocytes, or 5×10^5 APC with either 5×10^4 purified βgalTCR T cells or 5-25 $\times 10^4$ PBMCs, and Ag as indicated in a final volume of 200 μL. For proliferation assays the cells were pulsed with $[^3H]$ thymidine at 48 hr and counted at 72 hr post-stimulation. Cytokines were assayed at 48 hr post-stimulation by ELISA using antibodies and reagents from R&D Systems (Minneapolis, MN) or by cytometric bead array (CBA, BD Bioscience, San Jose, CA) per manufacturer's instructions. When indicated, mice were immunized by subcutaneous injection of 100 μg βgal peptide emulsified in complete Freund's adjuvant (CFA; Sigma, St. Louis, MO).

T cell and bone marrow transfers

Purified βgalTCR T cells were prepared from spleen and LN using anti-CD90.2 (Thy1.2) and/or anti-CD25 microbeads (Miltenyi, Auburn, CA) per manufacturer's protocol. The cells were washed and resuspended to a concentration of 5×10^7 cells/mL in PBS. Where indicated, the cells were labeled with 4 μM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37° C with mixing. Labeling was terminated by addition of 10 mL RPMI-1640 media supplemented with 10 % FCS. The cells were washed and resuspended to 2×10^7 cells/mL in saline. Recipient mice received the indicated number (10×10^6) of βgalTCR T cells via *ip* injection. βgalTCR bone marrow (BM) was extruded by femoral and tibial lavage with PBS. BM cells were PBS washed, RBC lysed, washed again, and resuspended in saline. BM recipient mice were irradiated (2 × 600 R, 4 hr interval) and received 4-5 × 10^7 cells in 100 μL *ip*.

In vivo treatment of βgalTCR T cell recipients

Groups of arrβgal and B10.A control mice were given an *iv* inoculation of 5×10^6 in vitro Ag-experienced, CFSE-labeled, resting βgalTCR T cells on day 0. Recipient mice were treated with 0.5 μg/mouse of pertussis toxin (PTx; Sigma, St. Louis, MO) on days 12 and 19, or given 20,000 U/mouse of IFNγ (R & D Systems, Minneapolis, MN) on days 12, 13, 14, 17, and 18. Control mice received saline only. Spleens were harvested for flow cytometry on day 28.

Flow cytometry

Spleen, thymus, or LN cell suspensions were prepared as described above except that the final resuspension was made in FACS buffer (PBS with 2 % FCS and 0.02 % sodium azide). 0.25-2.0 μL/10^6 cells of the appropriate fluorescent-labeled antibodies (BD Biosciences, San Diego, CA or eBioscience, San Diego, CA) were added and the suspensions incubated on ice for 30 min. The cells were then washed and resuspended in FACS buffer and analyzed on a FACSCalibur flow cytometer using CellQuest (BD Biosciences, San Jose, CA) or FlowJo (Tree Star, Ashland, OR) software.

Induction of autoimmune disease by adoptive transfer of T cells

Adoptive transfer for induction of EAU or brain inflammation was done with pooled, unfractionated βgalTCR splenocytes and LN cells stimulated with βgal peptide (0.5 μM), with or without IL-12 (5.0 ng/mL) or the combination of IL-1 (5 ng/mL), IL-6 (10 ng/mL), and TGFβ1 (2 ng/mL) to induce IL-17 production. At 48 hr post-stimulation, IL-2 was added (10 U/ml) and the βgalTCR T cells were cultured for an additional 6 hr. The cultures were washed, resuspended in PBS to 2×10^7 cells/mL, and inoculated at 5-20 × 10^6 βgalTCR T cells or splenocyte/lymphocyte cells per mouse, *ip*. Where indicated, T cells were isolated by Thy1.2 selection and activated with βgal peptide and irradiated B10.A × $Rag^{(-/-)}$ splenocytes at a splenocyte/T cell ratio of 10:1 for 48 hr. At the indicated times post-transfer, tissues were harvested, fixed in 10 % buffered formalin, paraffin embedded, sectioned (5 micron), and stained with hematoxylin and eosin. The slides were examined in a masked fashion with EAU scores of 0 (no disease) to 4 (complete loss of photoreceptor cells) based on histopathological changes to the retina (25). Brain inflammation was scored from 0 (none) to 4 (severe) based on histopathology (26).

Induction of autoimmune disease in double transgenic mice

Arrβgal or arrβgal × βgalTCR double Tg mice were given a single subcutaneous inoculation of 100 μg βgal protein emulsified in CFA with or without 0.5 μg PTx per mouse via *ip* injection. Eyes were harvested at 21 days post-immunization and analyzed for histopathology. The ability of soluble Ag and Ptx to induce disease was assayed by injecting mice *ip* with 200 μg βgal protein mixed with 0.5 μg Ptx. Eyes (from arrβgal × βgalTCR mice) and brains (from

GFAPβgal × βgalTCR mice used as controls) were harvested at 35 and 21 days post-immunization, respectively, and analyzed for histopathology.

Induction and analysis of autoimmune disease in lymphopenic mice

$CD25^+$ and $CD25^-$ fractions were isolated from βgalTCR spleen and LN cell suspensions using a $CD25^+$ selection kit and then enriched for $CD4^+$ cells by negative selection (Miltenyi). The cells were washed and resuspended in PBS to 5×10^6/ml and 3×10^5 cells were injected *iv* into $Rag^{(-/-)}$ or arrβgal × $Rag^{(-/-)}$ mice. The eyes were harvested at the indicated time points and analyzed as described above. Analysis of the delayed-type hypersensitivity (DTH) response to βgal was done by injection of βgal (50 μg in 10 μL) into the ear pinna as described previously (20).

Results

Analysis of βgalTCR T cells

Construction and initial identification of βgalTCR mice was made by PCR showing the presence of both the α and β TCR genes in genomic DNA (unpublished data). Unprimed βgalTCR mice exhibited a positive DTH response while single chain 3E9 α and β Tg mice did not (unpublished data). Analysis of βgalTCR splenocytes by flow cytometry showed 55-60 % of $CD3^+$ cells were $CD4^+V\beta10.1^+$, and more than 85 % of $CD4^+$ cells expressed Vβ10.1 (Fig. 1*A*). Further, comparison of naïve B10.A and βgalTCR $CD4^+VB10.1^+$ T cells showed similar expression of the activation markers CD25, CD44, CD45RB, and CD69, at levels consistent with the cells being phenotypically naïve (Fig. 1*B*). The number of $CD62L^{lo}$ cells from spleen was modestly increased in βgalTCR mice (Fig. 1*B*). In vitro proliferation assays demonstrated that βgalTCR splenocytes and/or lymphocytes responded to both βgal peptide and native βgal protein in a dose-dependent manner (Fig. 1*C*). Ag stimulation of unfractionated βgalTCR splenocytes produced significant amounts of IL-2 and IFNγ consistent with Th1 cells (27,28) (Table I), but little IL-4, IL-5, IL-10, and TNFα. IL-17 was also produced, consistent with the Th17 phenotype of autoimmune disease-inducing T cells (29,30). The Ag specificity of βgalTCR T cells was confirmed by the lack of cytokine production when stimulated with ovalbumin (OVA).

In vivo antigen recognition by naive βgalTCR T cells

Interactions in vivo between naïve βgal-specific T cells and retinal βgal were tested using purified, resting βgalTCR T cells from pooled spleen and LN. T cells were labeled with CFSE and transferred into βgal Tg and control mice. Labeled βgalTCR T cells were recovered from recipient LN and spleens ten days post-transfer and analyzed for evidence of Ag recognition. βgalTCR T cells transferred into ROSA26 mice, or control B10.A mice immunized with βgal peptide after transfer, showed clear evidence of Ag recognition compared to βgalTCR T cells transferred into normal B10.A recipients. Proliferation of βgalTCR T cells was indicated by dilution of CFSE in $V\beta10^+$ lymphocytes from ROSA26 and B10.A/peptide-inoculated mice (Fig. 2*A*) and in B10.A mice inoculated with whole βgal (Fig. 2*B*). Their expression of cell surface molecules associated with Ag recognition by T cells was also skewed toward the activated phenotype (increased CD44, CD69 and decreased CD45RB, CD62L; Fig. 2*A*). Conversely, βgalTCR T cells recovered from normal B10.A recipients, or from arrβgal mice, did not dilute their CFSE and maintained the naïve phenotype (Fig. 2*A*). Ag-specificity was further shown by inoculation with OVA, which did not induce CFSE dilution (Fig. 2*B*). βgalTCR T cells recovered from submandibular and cervical LN of recipient arrβgal mice were not phenotypically different than those recovered from inguinal and popliteal LN (unpublished data).

Development and function of βgalTCR T cells maturing in βgal Tg mice

Since short term residence of naïve βgalTCR T cells in arrβgal Tg mice offered little evidence of recognition of endogenous βgal, we asked if βgalTCR T cells maturing in arrβgal mice were altered in their development or ability to respond to βgal as measures of Ag recognition. Introduction of a more physiologically relevant number of developing βgal-specific T cells was achieved using BM grafting. βgalTCR mouse-BM was engrafted into irradiated B10.A or arrβgal mice, and T cell development and function were analyzed at 40 days post-transfer. FACS analysis of PBMC showed similar levels of βgalTCR T cell engraftment in both arrβgal and B10.A recipients (Fig. 3*A*). $CD4^+V\beta10^+$ T cells represented an average of 26.6 % and 29.9 % of all $CD3^+$ lymphocytes in the recipient B10.A and arrβgal mice respectively. A vast majority of these T cells are likely to be *bona fide* βgalTCR T cells since the endogenous Vβ10 TCR gene is expressed on only about 5 % of all T cells in control mice. βgalTCR T cells did not repopulate well in ROSA26 mice transferred with βgalTCR-BM as shown by the reduced percentage of $CD4^+V\beta10^+$ in $CD3^+$ lymphocytes (5.4 %). Unlike arrβgal mice, ROSA26 mice express βgal in the thymus, therefore this result is consistent with central tolerance and negative selection of maturing βgalTCR T cells. Analysis of $CD3^+V\beta10^+$ thymocytes from βgalTCR-BM engrafted arrβgal *vs.* B10.A mice showed nearly identical distributions of T cells into either CD4 or CD8 single positive, or CD4/CD8 double positive or double negative subsets (Fig. 3*B*), suggesting little or no central tolerance to βgal in arrβgal mice. Arrβgal mice receiving βgalTCR-BM did not develop EAU. Under these conditions, the results indicate that expression of βgal from the rod photoreceptor cell arrestin promoter had little effect on βgalTCR T cell development.

To determine if βgalTCR T cells that developed after βgalTCR-BM engrafting of βgal Tg recipients were altered by βgal in retinal photoreceptor cells, the phenotype, response, and effector functions of βgal specific T cells recovered from TCRβgal-BM engrafted recipient mice were compared at sixty days post-transfer. Cell surface activation makers were similar between naïve $CD4^+V\beta10^+$ T cells recovered from spleens of βgalTCR-BM engrafted arrβgal and B10.A mice as well as to control βgalTCR and B10.A mice (Fig. 3*C*). Similar results were observed in $CD4^+V\beta10^+$ cells recovered from LN (unpublished data). Splenocytes from βgalTCR-BM engrafted arrβgal and B10.A mice exhibited similar dose-dependent proliferative responses to βgal peptide (Fig. 3*D*).

To compare the development of memory βgalTCR T cells in mice whose naïve T cells matured with or without βgal expressed in photoreceptor cells, βgalTCR-BM recipient mice or control mice were immunized with βgal peptide, and splenocytes were analyzed twenty-one days post-immunization. Immunized βgalTCR-BM recipient arrβgal mice and B10.A mice showed similar increases in CD44 levels and decreases in CD62L levels (Fig. 3*E*), indicating that the development of the effector memory T cell phenotype was not affected by the presence of βgal in photoreceptor cells. In summary, these results suggested that the presence of βgal in photoreceptor cells had little effect on the phenotype, response, or effector functions of naïve or memory βgalTCR T cells. Further, no spontaneous EAU, or EAU induction by immunization with βgal/CFA, was observed in any of the βgal Tg mice grafted with βgalTCR-BM.

Manipulation of antigenic ignorance

It has been proposed that a lack of Ag presentation of retina-derived Ags in peripheral LN limits the generation of peripheral tolerance, leading to increased susceptibility to EAU (10). Since Ag-experienced T cells are widely thought to have increased ability to detect and respond to Ag, adoptive transfer of Ag-experienced, CFSE-labeled, resting βgalTCR T cells, and treatments to promote Ag presentation of retinal βgal, were used to probe for evidence of peripheral retinal Ag recognition (Fig. 4). Although none of the mice receiving the βgalTCR T cells along with IFNγ or PTx treatment developed EAU, there were small, but significant,

Ag-dependent changes in: 1) recovery of CFSE-labeled βgalTCR T cells from spleen, 2) their percentage of all $CD4^+V\beta10^+$ cells and, 3) the level of CFSE fluorescence in $CFSE^+$ cells recovered from IFNγ-treated, or PTx-treated arrβgal *vs.* B10.A recipients. These parameters were unchanged in control comparisons of saline-treated arrβgal *vs.* B10.A mice and IFNγ *vs.* saline-treated B10.A mice. These results suggest that manipulations designed to increase Ag presentation produced a limited, non-pathogenic, T cell recognition of retinal βgal.

Susceptibility of single and double Tg mice to EAU induction

Several TCR Tg mouse models of autoimmune disease have used double Tg mice in which both the target Ag and Ag-specific T cells are present (9,10,31,32). Accordingly, we generated arrβgal × βgalTCR double Tg mice and compared the ability to induce EAU in these double Tg mice versus single Tg arrβgal mice. Arrβgal mice were moderately susceptible to induction of EAU by immunization with βgal in CFA, followed by PTx given *iv* (Table II). Conversely, double Tg arrβgal × βgalTCR mice were highly resistant to the same immunization protocol, and did not develop spontaneous EAU when aged up to 14 months in SPF conditions.

Adoptive transfer of βgalTCR T cells

Using βgal peptide to activate pooled spleen and LN cells from βgalTCR mice in vitro led to potent activation as shown by cytokine production (Table I). After two days, $5\text{-}20 \times 10^6$ viable cells were transferred into the βgal Tg mice. Preliminary studies showed that GFAPβgal mice, which express βgal in CNS astrocytes, developed CNS infiltrates and lesions following adoptive transfer of βgalTCR T cells (unpublished data). Therefore, they were used as positive controls for the activity of the various preparations of βgalTCR T cells that follow. None of 18 arrβgal mice developed EAU, but 4 of 8 GFAPβgal mice developed inflammatory lesions in the brain (Table III). The large difference in susceptibility of the retina as compared to brain was unexpected, as the concentration of Ag in retina is much higher than in brain; i.e. the 150 ng of βgal/retina is concentrated in 3 μL of retinal tissue whereas the 175 ng in brain is contained in approximately 500 μL of tissue.

To determine if more potent activation of the T cells would enhance disease induction, cultures were activated in the presence of agents known to promote development of pathogenic $CD4^+$ T cells in EAU, including IL-12 (33), which failed to promote EAU induction (Table III). Inclusion of IL-1, LPS, CpG or PTx in the cultures during activation also did not promote disease in arrβgal recipients (unpublished data). Since Th17 cells have been identified as important mediators of EAU (34), βgalTCR T cells were cultured with the combination of IL-1, IL-6, and TGFβ1 to induce differentiation of IL-17 secreting Th17 cells (35). A single Ag activation cycle in the presence of these three cytokines gave a moderate level of IL-17 production, while a second cycle induced very high levels of IL-17 production (Fig. 5*A*). Subsequent activation cycles in the presence of the IL-17 inducing cytokines did not further increase IL-17 levels, but the inducing cytokines were necessary to maintain IL-17 production (unpublished data). The combination of cytokine supplements had little effect on IL-2 production compared to cultures stimulated without cytokines (Fig. 5*B*). TNFα and IFNγ levels were also assayed since these cytokines are associated with EAU pathogenesis (36,37), but they were unaffected (unpublished data). While the IL-17-secreting phenotype was induced in the cytokine-treated βgalTCR T cells, there was no increase in the frequency of EAU in arrβgal mice compared to mice that received βgalTCR T cells stimulated without cytokines. Only one mouse developed mild disease in the peripheral retina (Fig. 6*B* and Table III). These same IL-17-induced βgalTCR T cells were significantly pathogenic in the GFAPβgal mice.

Differential induction of autoimmune disease in βgalTCR × βgal double transgenic mice

Since a recent study reported that soluble Ag along with PTx, in conjunction with transferred, Ag-specific $CD4^+$ T cells, was a potent inducer of autoimmune disease in mice that expressed

the Ag in the eye (38), the ability of PTx and soluble βgal to induce EAU in the arrβgal × βgalTCR double Tg mice was analyzed. Only 1 of 15 double Tg mice developed disease using this protocol (Fig. 6*C-D* and Table IV). To confirm the function of the βgalTCR Tg T cells in double Tg mice, GFAPβgal × βgalTCR mice were tested concurrently and found to develop significant inflammatory infiltrates (Table IV). PTx alone did not induce autoimmune disease in the susceptible GFAPβgal × βgalTCR mice suggesting that Ag availability was a limiting factor.

Lymphopenia-induced autoimmune disease

LIP has been associated with acquisition of an activated T cell phenotype, and inflammatory disease in murine models (39-42). A small number (3×10^5) of $CD25^-$ T cells isolated from βgalTCR × $Rag^{(-/-)}$ mice were transferred into arrβgal × $Rag^{(-/-)}$ mice and a control group of $Rag^{(-/-)}$ mice. Eyes harvested between 2 and 11 weeks post-transfer showed a slowly progressive, but relentless destruction of the entire photoreceptor layer (Fig. 7) by a process that was minimally inflammatory, but highly destructive (Fig. 6*E-G*). Transfer into $Rag^{(-/-)}$ mice lacking retinal βgal expression gave no EAU (0/12), nor did transfer of an equivalent number of $CD25^+$ T cells from βgalTCR × $Rag^{(-/-)}$ donors into arrβgal × $Rag^{(-/-)}$ recipients (0/4).

Retinal Ag affected the recovery of βgalTCR T cells

In addition to the retinal destruction associated with LIP by naïve CD25-depleted βgalTCR T cells in the arrβgal × $Rag^{(-/-)}$ recipients, evidence for other Ag-dependent effects on the number, phenotype, and function of the recovered cells was sought. Approximately twice as many βgalTCR T cells were recovered from $Rag^{(-/-)}$ recipients compared to arrβgal × $Rag^{(-/-)}$ recipients (Table V). Each of the populations contained similar portions of $CD25^+Foxp3^+$ T cells, approximately 4 %, but the number of cells with a $CD25^+Foxp3^+$ Treg phenotype was greater in the $Rag^{(-/-)}$ recipients without βgal. $CD25^+Foxp3^+$ T cells could be detected at three weeks post-transfer (unpublished data) and were similarly present in both types of recipients at ten weeks post-transfer (Fig. 8*A*).

Cytokine production by recovered βgalTCR T cells was substantially altered by their residence in both types of lymphopenic recipients relative to normal βgalTCR T cells, but the effect of the presence of βgal was minimal (Fig. 8*B*). The level of cytokine production was numerically lower, in most cases, in cells recovered from arrβgal × $Rag^{(-/-)}$ recipients, but no single significant difference from the $Rag^{(-/-)}$ recipients was found. In another experiment, the $CD25^+$ cells from the lymphopenic recipients of CD25-depleted βgalTCR T cells were isolated and tested for their ability to inhibit Ag-stimulated IL-2 production by naïve βgalTCR T cells. The $CD25^+$ cells had little ability to inhibit the IL-2 production of fresh βgalTCR T cells, and also produced little IL-2 upon stimulation with Ag/APC (Fig. 8*C*).

Tregs derived from the T cell inoculum

We previously demonstrated the presence of an uncharacterized, transferable regulatory activity in the arrβgal mice (20), and showed above that the arrβgal mice were resistant to adoptive transfer of EAU by the βgalTCR T cells. Since $Rag^{(-/-)}$ mice do not have conventional, endogenous $CD4^+25^+Foxp3^+$ T cells, we tested the possibility that arrβgal mice on the $Rag^{(-/-)}$ background might be more susceptible to EAU induced by adoptive transfer of in vitro activated βgalTCR T cells. $Thy1.2^+$ cells were isolated from pooled βgalTCR spleen and LN, activated for 48 h with Ag presented by irradiated splenic APC from $Rag^{(-/-)}$ mice, and transferred to arrβgal and arrβgal × $Rag^{(-/-)}$ mice. The modified activation protocol generated T cells that induced a low, but significant incidence of EAU, with minimal severity. However, there was no difference in the incidence and severity of EAU between the two groups of

recipients (Fig. 9, left) suggesting that endogenous Tregs had a limited role in preventing pathogenesis.

Since the EAU induced by naïve $CD25^-$ T cells in the arrβgal × $Rag^{(-/-)}$ recipients was slow to develop, we asked if activated βgalTCR T cells that had been depleted of $CD25^+$ cells prior to activation would induce EAU more rapidly and be more pathogenic in arrβgal × $Rag^{(-/-)}$ recipients. CD25-depleted, pre-activated βgalTCR T cells were much less pathogenic in lymphopenic recipients than were the naïve CD25-depleted cells. Further, the normal arrβgal recipients remained resistant to EAU induction using either naïve or activated, CD25-depleted βgalTCR T cells (Fig. 9). These results suggested the possibility that Tregs were present in the T cell inoculum. In preliminary studies, we noted that in vitro activation of T cells from βgalTCR mice yielded an increase in the population of $Foxp3^+$ T cells, which could be Tregs. As shown in Fig. 10, $CD4^+25^+Foxp3^+$ cells were indeed generated from Thy1.2-selected, CD25-depleted βgalTCR T cells by Ag stimulation and present in the inocula, where they could contribute to the resistance to EAU. Since cells with the Treg phenotype appeared within 48 h of activation, proliferation of a small number of contaminating Tregs could not account for the 10-fold increase.

Discussion

We have created TCR-Tg mice (βgalTCR) specific for the neo-self Ag βgal in order to investigate factors leading to autoimmune pathogenesis resulting from recognition of CNS Ags that originate from retinal photoreceptor cells (neurons) by naïve and activated class II MHC-restricted $CD4^+$ T cells. Although spontaneous autoimmune disease was not observed in arrβgal × βgalTCR double Tg mice, our results demonstrated that βgalTCR T cells were capable of in vivo CNS antigen recognition, but autoimmune disease in retina was found under a very limited set of activation conditions. Only a single strategy, lymphopenia-induced lymphoproliferation of CD25-depleted, naïve βgalTCR T cells, generated conditions that resulted in a high incidence of severe EAU in arrβgal mice.

T cells from TCR-Tg mice that recognize either endogenous self-Ags or neo-self Ags have been instrumental in advancing the understanding of the multifaceted processes of self-tolerance and autoimmunity (31). Both regulatory and immunopathogenic mechanisms were recently reported in two related models of retinal Ag-specific, TCR-Tg mice. Membrane-bound retinal expression of HEL under control of the IRBP promoter, in conjunction with the 3A9 TCR-Tg T cells specific for HEL, gave a high incidence of spontaneous, severe EAU (10). The absence of Ag presentation, due to the sequestration of Ag in a neural environment, was proposed to limit the generation of peripheral T cell anergy, and Tregs were not found. These conditions were proposed to be responsible for the high degree of spontaneous HEL-targeted retinal autoimmune disease in the HEL/3A9 double Tg mice, despite the significant level of negative selection due to *aire*-dependent thymic expression of HEL (10). In a related model, HEL with a membrane anchor was expressed on the retinal rhodopsin promoter and studied in double Tg mice also made with 3A9 T cells (9). In this model, severe, spontaneous disease was also found in the double Tg mice, but there was also clear evidence of substantial negative selection due to thymic HEL expression. The authors proposed that the robust disease was due to low affinity, HEL-specific T cells that escaped thymic deletion, although the source of these cells in the TCR-Tg T cell population was not identified. The tolerance induced by HEL expression in single Tg mice was proposed to be largely due to negative selection. It is possible that the photoreceptor cell damage associated with this form of HEL Tg expression in the photoreceptor cells altered the local environment, and subsequent immunological response to the Ag (9).

The results from both of these models differ from ours in several ways. First, we found no evidence for thymic βgal expression. There were minimal differences in the number, survival, phenotype, or qualitative responses of βgalTCR T cells that resided and matured in B10.A and arrβgal hosts that would be associated with thymic expression. Conversely, the low level systemic and thymic expression of βgal in ROSA26 mice strongly affected the development and function of βgalTCR T cells. Further, whether by βgalTCR-BM transfer, or transfer of mature, naïve βgalTCR T cells, or analysis of T cells from double Tg mice, there was little evidence of recognition by T cells of photoreceptor cell-derived βgal in untreated recipients, as no reliable differences in cell-surface phenotype or function were detected. Secondly, the double Tg mice were highly resistant to spontaneous EAU, even though the TCRβgal T cells were highly pathogenic under LIP conditions.

Findings from CNS studies consistent with our results were found using double Tg mice that express OVA in brain oligodendrocytes and generate OVA-specific OT-II TCR Tg T cells. There was no evidence for recognition of cognate Ag by the T cells and no spontaneous autoimmune disease (43). A naïve phenotype was also observed in influenza virus hemagglutinin (HA) Ag-specific $CD4^+$ T cells derived from HA-specific TCR-Tg mice crossed with mice expressing HA in glial cells (44). The lack of spontaneous autoimmune disease in arrβgal × βgalTCR double Tg mice also bears a superficial resemblance to experimental autoimmune encephalomyelitis (EAE) models using various well-known myelin basic protein (MBP)-specific TCR Tg mice, especially those whose T cells are specific for the MBP 1-11 epitope in which administration of adjuvants is required for disease onset (32). Several additional important factors further distinguish our results from those. First, the use of adjuvants did not reverse the resistance to disease induction in arrβgal mice. Second, compared to the widely expressed MBP, there is very little total βgal in arrβgal mice (300 ng/mouse) and its expression is highly restricted to photoreceptor cells of the retina and to subnanogram levels in the pineal gland. Third, the MBP epitopes are also contained in the golli form of the protein that is highly expressed, even in cells of the immune system including dendritic cells (45,46). This leads to selection of the T cell repertoire, resulting in the generation of pathogenic and regulatory T cells that recognize MBP (47-49). Fourth, the βgal epitope is immunodominant, implying the efficient generation and persistence of stable peptide-MHC class II complexes. It has been reported that MBP 1-11-MHC class II complexes are unstable (50) and that there is competition for MHC class II binding between MBP 1-11 and flanking golli and MBP sequences (51). Finally, the CNS has clear pathways of lymphatic drainage (2,3), whereas the retina contains only occasional $LYVE\text{-}1^+$ microglia (52), which does not constitute draining lymphatics in the normal retina.

The mechanisms that yield the remarkable resistance to autoimmune disease to an Ag expressed in photoreceptor cells appear to be multiple. Evidence supporting the antigenic ignorance hypothesis by Lambe et al. (10) is found from results showing no differences between naive βgalTCR T cells existing in βgal Tg versus B10.A mice, suggesting diminished peripheral access to the Ag.

However, βgalTCR T cells clearly crossed the blood-retinal barrier, gained access to their target Ag in photoreceptor cells, and created the severe retinal pathology shown in lymphopenic recipients. Activated T cells, even with no particular specificity for an Ag within the retina or brain, penetrate the blood-brain barrier and the blood-retinal barrier (53,54). The outcome of that penetration appears to result from an important, but undefined, local factor in susceptibility. The partial reversal of this state of ignorance by systemic treatment with IFNγ and PTx found in the arrβgal mice is further consistent with maintenance of antigenic ignorance. However, this state should leave the mice, as proposed by Lambe et al. (10), hypersensitive to attack by adoptive transfer of activated, autoreactive T cells. This is clearly not the case with the arrβgal Tg mice. While attack on βgal^+ astrocytes by the T cells was promoted by in vitro activation

protocols, no significant susceptibility was observed in the arrβgal mice. Obviously, there remains an important local barrier to pathogenesis in these mice that is unrelated to sequestration, as the retina appears to pose no barrier to activated T cells (54).

In a recent report using the MBP model for EAE, transfer of naive, TCR Tg, MBP-specific CD4 T cells into $Rag^{(-/-)}$ or $TCR\alpha^{(-/-)}$ deficient mice was found to rapidly produce severe signs of EAE within 5 days of transfer (42). Pre-transfer of normal spleen cells provided sufficient regulatory T cells to prevent disease. In our LIP strategy, it is worth noting that disease required 8-9 weeks to develop in the arrβgal × $Rag^{(-/-)}$ mice, but then progressed to total destruction of the target cells in the retina. Recovery of the T cells from $Rag^{(-/-)}$ and arrβgal × $Rag^{(-/-)}$ recipient mice showed that their Ag-stimulated cytokine production was similar, but much different from naïve βgalTCR T cells that had not undergone LIP.

While adoptive transfer of activated, autoreactive $CD4^+$ T cells is a common strategy to induce experimental autoimmune disease in the retina, use of Tg mice that express a target Ag in photoreceptor cells, in conjuction with $CD4^+$ TCR Tg T cells, revealed the presence of multiple mechanisms of resistance to EAU. Transfer of naive, CD25-depleted, βgalTCR T cells into arrβgal mice on the $Rag^{(-/-)}$ background was the only procedure we have found to date that induced a high frequency of severe retinal disease. Since $Rag^{(-/-)}$ recipients have no endogenous Tregs that can limit autoimmunity, it suggests that such Tregs may be another source of resistance to EAU in arrβgal mice. Surprisingly, CD25-depletion of the donor βgalTCR mice prior to harvesting the T cells, and then depleting the $CD25^+$ cells again prior to in vitro activation with APC and Ag still did not confer significant pathogenicity, even in lymphopenic recipients. Since the activation of CD25-depleted T cells prior to transfer induced $Foxp3^+$ expression, additional evidence for peripheral induction of Tregs was found. In light of these results, we propose that the limitation on retinal disease acted via at least two possible mechanisms: 1) the CD25-depleted donor T cell population was still able to give rise to regulatory cells and 2) interaction of the transferred T cells with a local retinal cell population limited disease induction and/or progression. LIP-induced activation and in vitro Ag/APC stimulation both resulted in the appearance of $CD4^+CD25^+Foxp3^+$ T cells, but significantly more disease was found in the lymphopenic recipients. We are pursuing the possibility that regulatory cells induced by Ag stimulation are more protective than those that appear during LIP.

Acknowledgements

The authors thank Heidi Roehrich for histology, Thien N. Sam for technical assistance, and Kathleen M. Pierson for animal care and genetic analysis. The authors have no conflicting financial interest.

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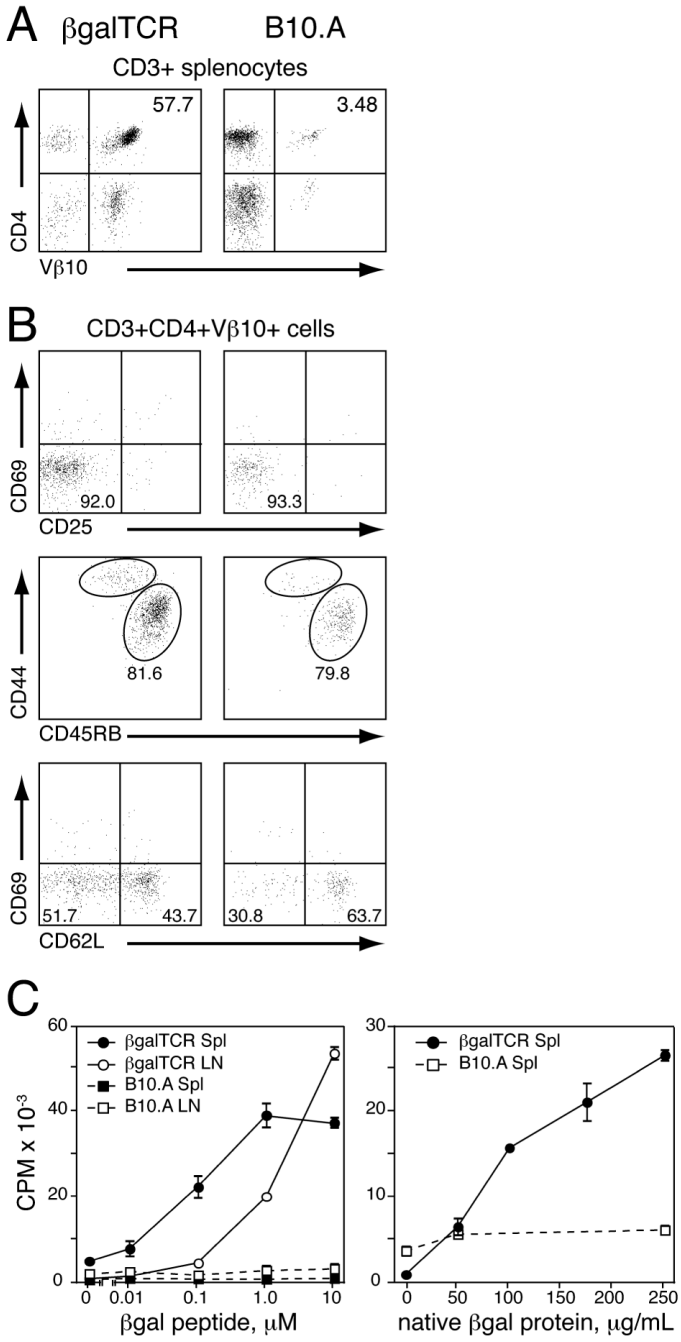

Figure 1.

Analysis of phenotype and Ag response of T cells from βgalTCR Tg mice. (*A*) FACS analysis of $CD3^+$ splenocytes from B10.A control (right) and βgalTCR Tg (left) mice. Percentage of cells with a specific phenotype is indicated. (*B*) FACS analysis of $CD3^+CD4^+V\beta10^+$ splenocytes from naïve B10.A control (right) and βgalTCR Tg (left) mice for cell surface activation markers. (*C*) Proliferative response of control B10.A and TCRβgal Tg mouse lymph node cells and/or splenocytes to βgal peptide (left) or native βgal protein (right). Values shown are the mean of three animals, each done in triplicate, with error bars indicating standard error of mean (SEM).

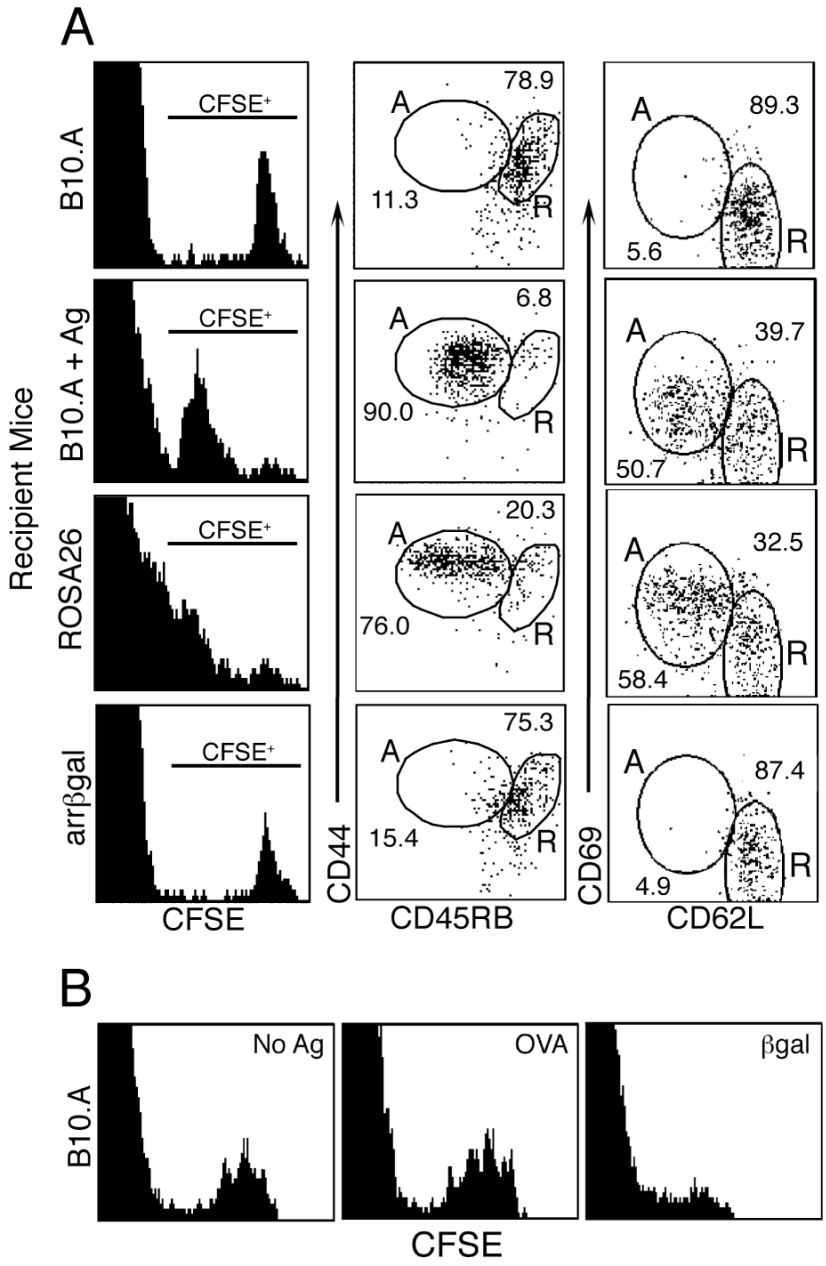

Figure 2.

Analysis of Ag recognition in vivo by adoptively transferred, CFSE-labeled naïve βgalTCR T cells. (*A*) Representative FACS analysis of Vβ10$^+$ lymphocytes recovered from recipient mice 10 days post-transfer for CFSE dilution and the indicated cell surface molecules. The percentage of lymphocytes having a resting/naïve (R) or an activated (A) phenotype is indicated. B10.A + Ag = 100 μg βgal peptide given *ip* to B10.A recipient mice three days post-T cell transfer. (*B*) Analysis of Ag specificity. FACS analysis of Vβ10$^+$ lymphocytes from spleens of B10.A mice that received 100 rads irradiation prior to transfer of naive, CFSE-labeled βgalTCR T cells. Cells were harvested 14 days post-transfer. Recipients were given *ip* injections of saline (No Ag); 1 mg OVA; or 300 μg native βgal three days post-T cell transfer.

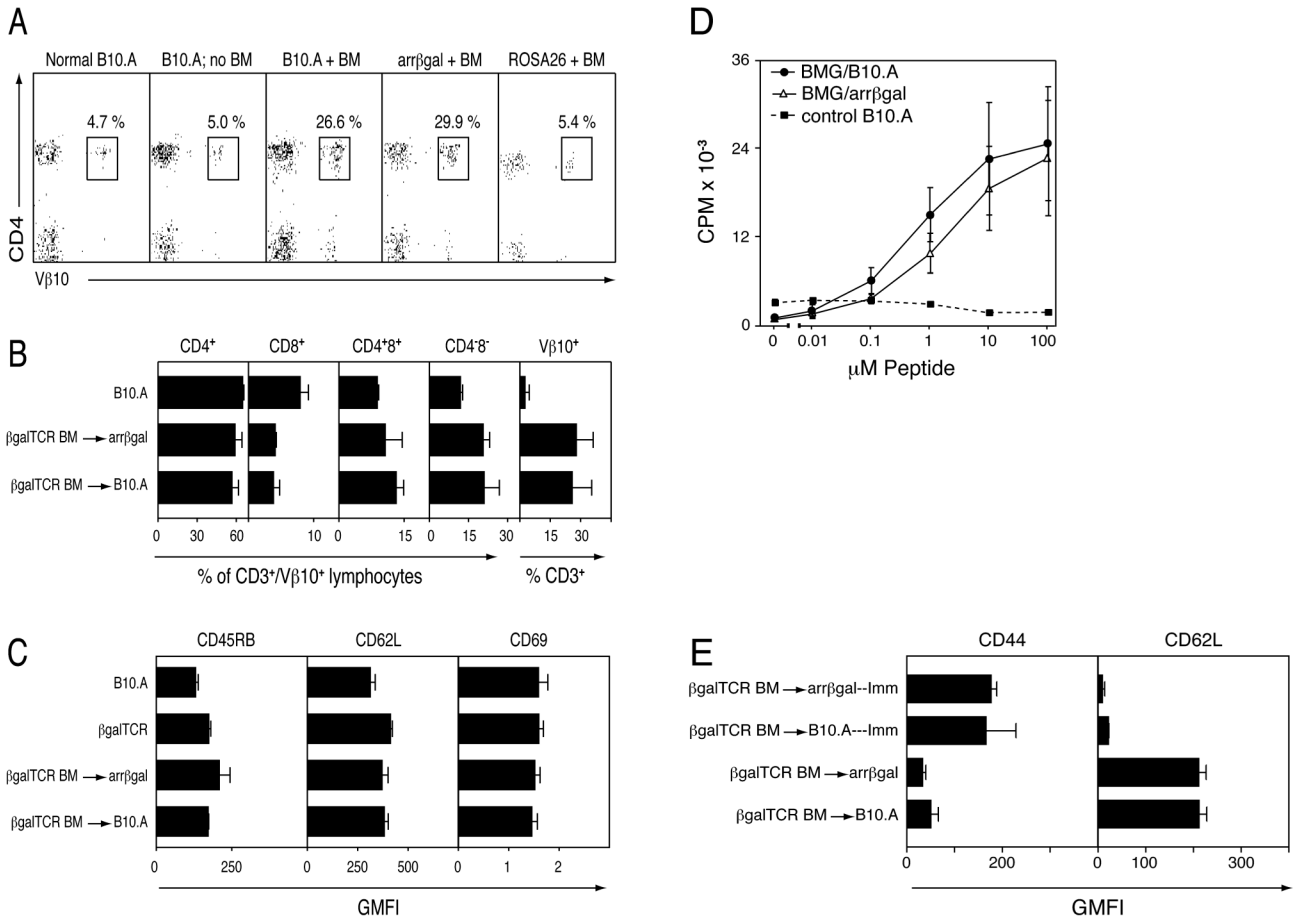

Figure 3.

Analysis of βgalTCR T cells derived from βgalTCR-BM engrafted mice. (*A*) Representative FACS analysis of $CD3^+$ lymphocytes from peripheral blood for percentage of $CD4^+V\beta10^+$ cells 40 days post-BM transfer. The normal B10.A mice were not irradiated or grafted; all other groups were irradiated and given βgalTCR-BM where indicated. (*B*) FACS analysis of $CD3^+V\beta10^+$ thymocytes from control (B10.A) and βgalTCR-BM engrafted mice for T cell subset distribution. Also shown is the percentage of $CD3^+$ thymocytes expressing Vβ10. Assays performed 40 days post BM transfer. (*C*) FACS analysis of $CD4^+V\beta10^+$ splenocytes for the indicated cell surface activation marker from B10.A, βgalTCR, and βgalTCR-BM engrafted mice 60 days post BM transfer. (*D*) Proliferative response of splenocytes from βgalTCR-BM engrafted mice to βgal peptide. Values represent the mean of three animals, each done in triplicate, with error bars indicating SEM. (*E*) FACS analysis of $CD4^+V\beta10^+$ splenocytes for cell surface markers of memory phenotype in immunized and non-immunized βgalTCR-BM engrafted recipient mice. For *B*, *C*, and *E*, bars indicate the mean for all animals in each group ($n \geq 3$) with error bars indicating SEM.

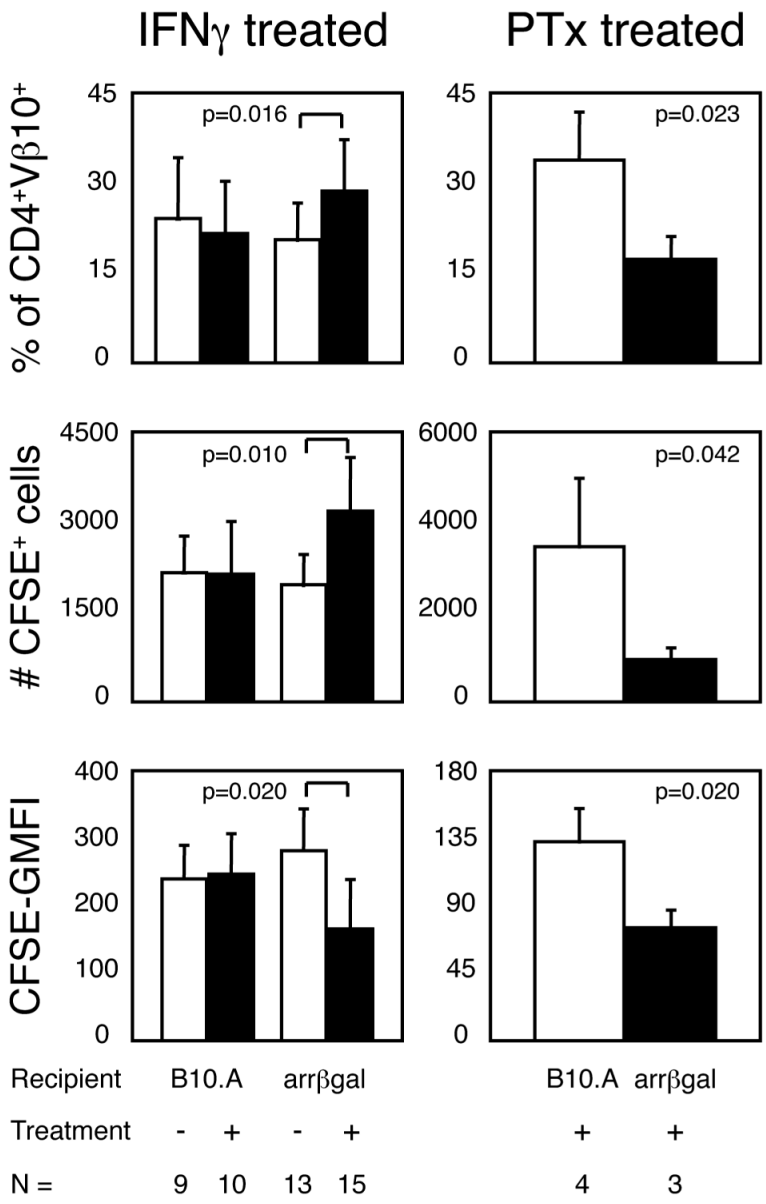

Figure 4.

In vivo administration of IFNγ or PTx leads to Ag-dependent changes in βgalTCR T cells. Resting, CFSE-labeled βgalTCR T cells were transferred into mice that were treated with IFNγ (left) or PTx (right) as described in materials and methods. Bars indicate mean value for all animals in each group with error bars indicating SEM. p values determined by student's t test.

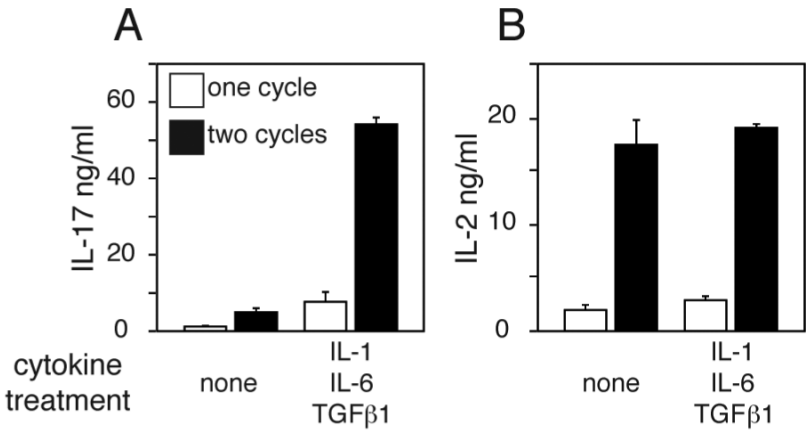

Figure 5.

Induction of IL-17 production by βgalTCR T cells. Production of IL-17 (*A*) and IL-2 (*B*) following one or two rounds of Ag/APC stimulation with indicated cytokine treatments as describe in Material and Methods. Error bars indicate SEM.

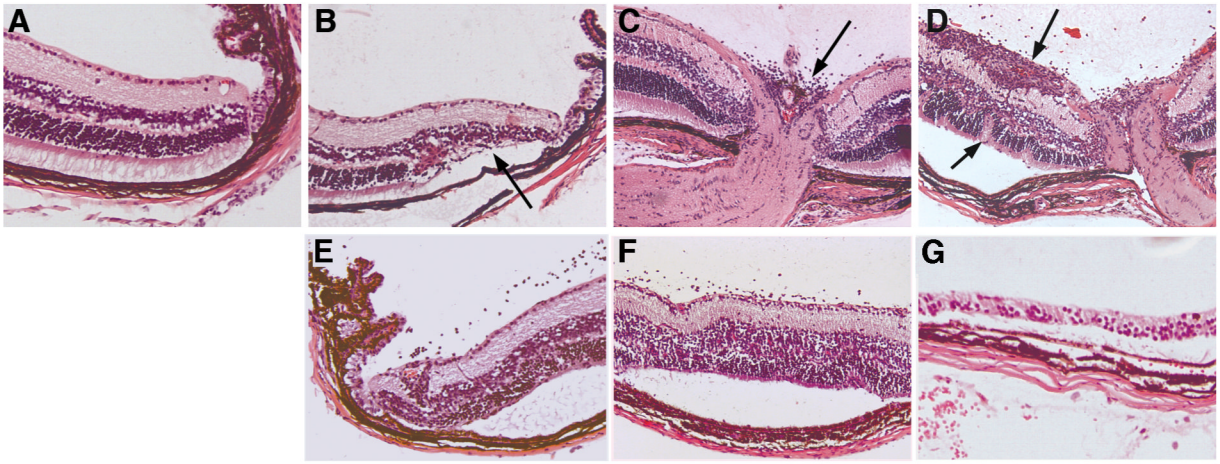

Figure 6.

Representative photomicrographs of autoimmune pathology in arrβgal retina induced by βgalTCR T cells. (*A*) Normal retina. (*B*) Retina 21 days post-transfer of 5×10^6 βgalTCR T cells after 4 stimulation cycles under IL-17-inducing culture conditions. Modest loss of peripheral photoreceptor cells was evident after active inflammation subsided (arrow). (*C-D*) Retinas from an arrβgal × βgalTCR double Tg mouse 35 days after IV inoculation of 0.5 μg PTx and 200 μg βgal showing modest infiltration of the optic nerve head (*C*, arrow) and substantial active infiltration of the inner layers of the retina plus disruption of the photoreceptor cell layer (*D*, arrows). (*E-G*) arrβgal × $Rag^{(-/-)}$ retinas at 49 (*E*), 62 (*F*), and 75 (*G*) days post-transfer of 3×10^5 naïve $CD25^-$ βgalTCR T cells showing progressive loss of photoreceptor cells. (*A-G*), 100 X magnification.

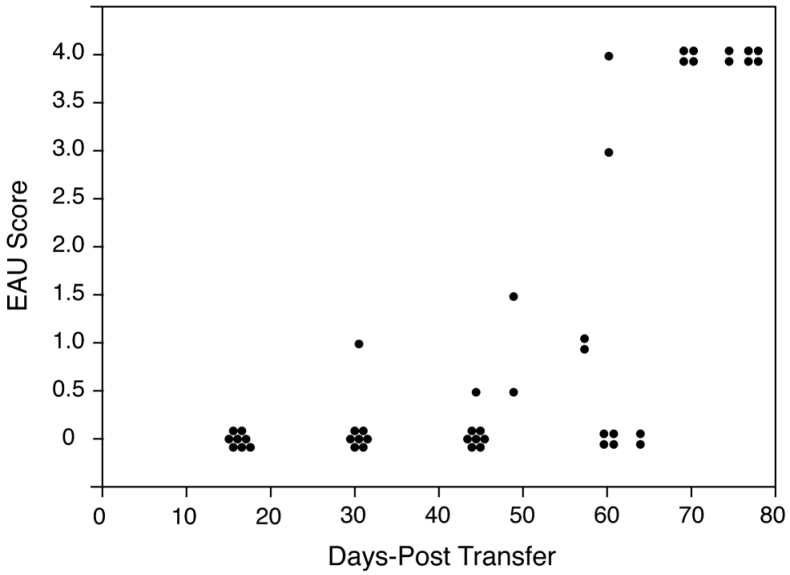

Figure 7.

LIP leads to EAU in arrβgal mice. 3×10^5 $CD25^-$ βgalTCR T cells were transferred into arrβgal × $Rag^{(-/-)}$ recipients and the eyes were harvest at the indicated time post-transfer and scored for EAU. Each dot represents one eye.

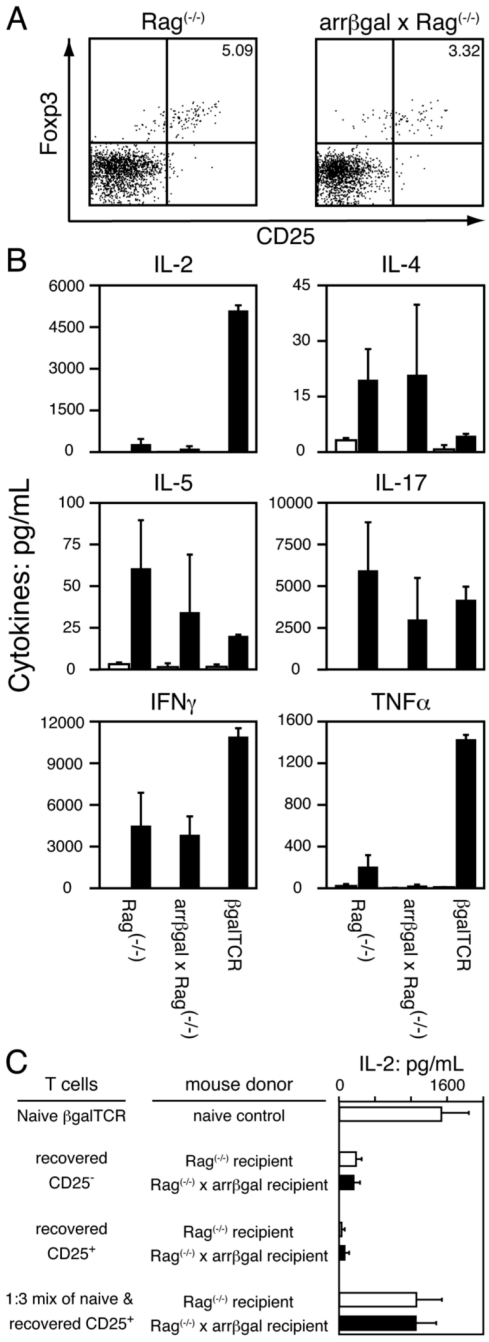

Figure 8.

Analysis of T cells recovered from lymphopenic recipient mice reflects the contributions of Th1, Th2, and regulatory T cells. Spleen and LN cells were harvested 9-11 weeks post-transfer of $CD25^-$ βgalTCR T cells into $Rag^{(-/-)}$ and arrβgal × $Rag^{(-/-)}$ mice. (*A*) Representative FACS plots of $CD4^+V\beta10^+$ T cells recovered from transferred $Rag^{(-/-)}$ and arrβgal × $Rag^{(-/-)}$ mice showing similar proportions of cells that are $CD25^+Foxp3^+$. (*B*) Analysis of cytokine production by 5×10^5 pooled, Ag-stimulated spleen and LN from normal βgalTCR Tg mice and lymphopenic mice transferred with $CD25^-$ βgalTCR T cells. Solid bar = with Ag, open bar = no Ag. (*C*) IL-2 production by 1×10^5 $CD25^+$ T cells and $CD25^-$ T cells recovered from

$Rag^{(-/-)}$ and arrβgal × $Rag^{(-/-)}$ recipient mice, and 1×10^5 recovered $CD25^+$ T cells mixed with 3.5×10^4 naïve βgalTCR T cells upon stimulation with Ag and APC.

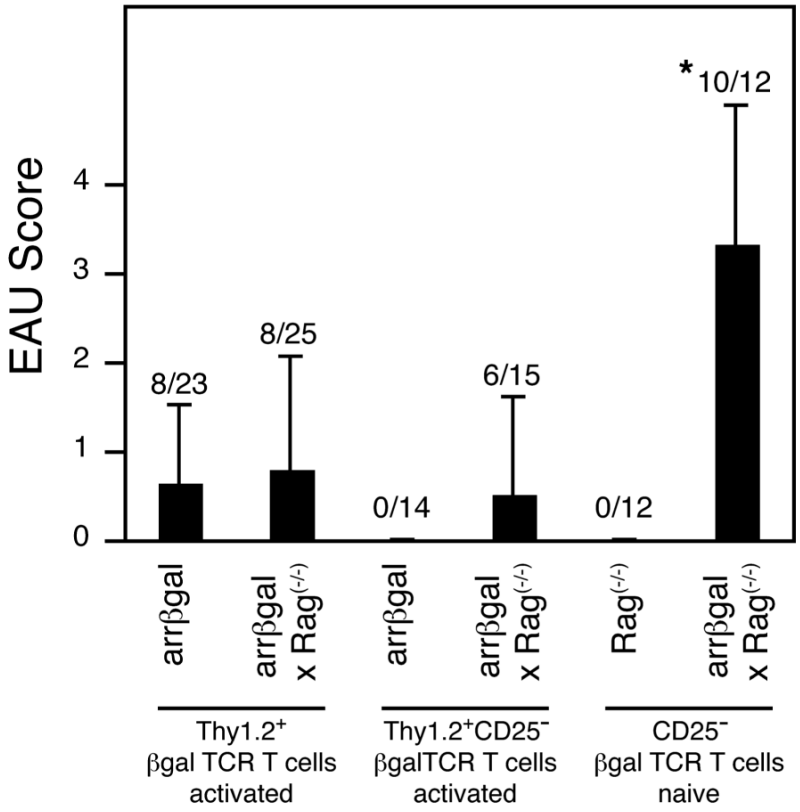

Figure 9.

Induction of EAU by adoptive transfer of βgalTCR T cells. The T cells were activated and/or selected as indicated and transferred into indicated recipient mice. * = $p < 0.005$ compared to all other groups for both incidence (Fisher's exact test) and severity (Mann-Whitney test).

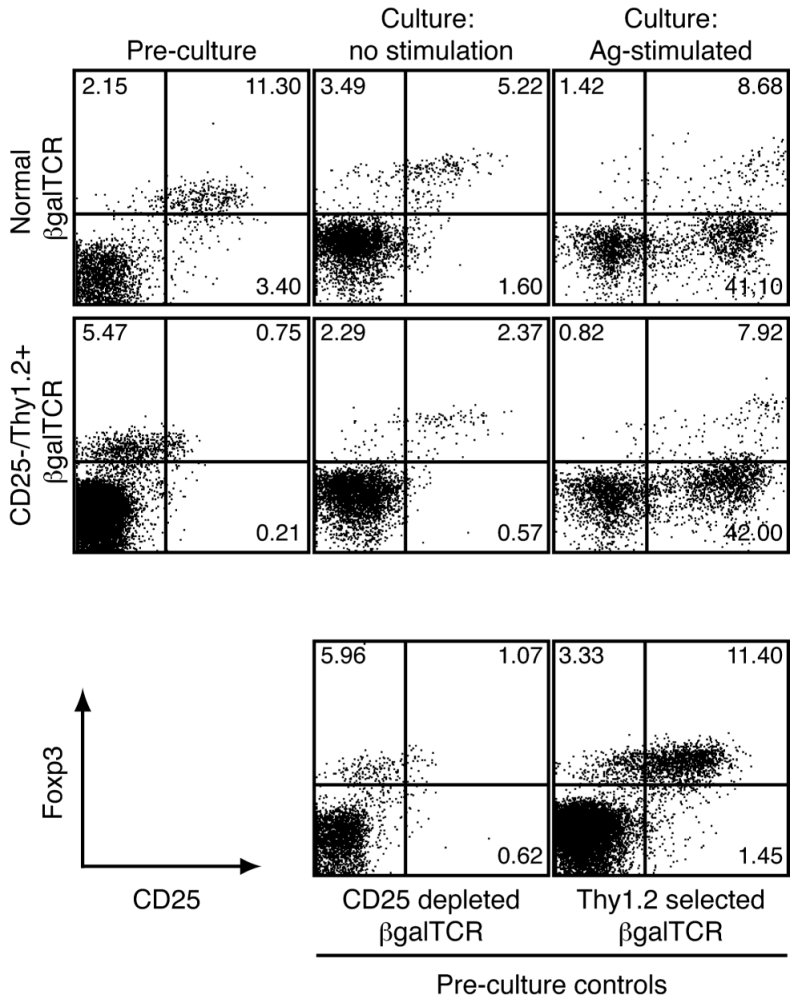

Figure 10.

Production of T cells with the Treg phenotype in Ag-stimulated cultures. Normal and selected βgalTCR T cells were analyzed for $CD25^{+}Foxp3^{+}$ cells prior to culturing and after 48 h in culture with or without Ag. FACS plots are gated on $CD4^{+}$ lymphocytes.

Table I

Cytokine production by Ag stimulated βgalTCR splenocytes

	Cytokine, pg/mL[a]						
Ag[b]	**IL-2**	**IL-4**	**IL-5**	**IL-10**	**IL-17**	**INFγ**	**TNFα**
none	5.6 ± 2.9	0.7 ± 1.0	2.5 ± 1.6	BD[c]	4.1 ± 3.6	1.7 ± 0.8	15.2 ± 8.5
OVA	6.8 ± 1.0	BD[c]	2.1 ± 0.5	ND[c]	21.1 ± 23.0	14.5 ± 1.4	178 ± 21
βgal	3470 ± 2275	5.0 ± 2.4	31 ± 15	460 ± 70	6605 ± 2195	8071 ± 3835	1434 ± 38

[a] ± standard deviation.

[b] 100 ug/ml ovalbumin, 10 μM βgal peptide.

[c] BD = below detection, ND = not determined.

Table II

Inhibition of EAU induction in arrβgal × βgalTCR double Tg mice

	arrβgal		arrβgal × βgalTCR		
Immunization	**incidence**	**severity**	**incidence**	**severity**	**P value[a]**
βgal + CFA	0/8	0	0/15	0	
βgal + CFA + PTx	12/36	0.5, 0.5, 0.5, 1, 1, 1, 2, 2, 2.5, 3, 3.5, 4	0/12	0	0.023

[a]Fisher's exact test: 2-tailed; arrβgal vs. arrβgal × βgalTCR

Table III

arrβgal mice resisted induction of EAU by adoptive transfer of pathogenic T cells

	Recipient Mice				
	arrβgal[a]		GFAPβgal[b]		
Cells Transferred[c]	incidence	severity	incidence	severity	P value
untreated	0/18	0	4/8	0.5, 1.0, 1.5, 2.0	0.0047[d]
IL-12 (exogenous)	0/17	0	ND		
IL-17 (induced)	1/23	1.0	5/13	0.5, 1.0, 1.0, 2.0, 3.5	0.0161[d]

[a] EAU 21 days post-transfer.

[b] brain inflammation 5-7 days post-transfer.

[c] Unfractionated spleen and LN cells plus Ag and indicated cytokine treatment.

[d] Fisher's exact test; 2-tailed; arrβgal vs. GFAPβgal.

Table IV

ArrβGal × βgalTCR double Tg mice were resistant to EAU

	Recipient Mice				
	arrβgal × βgalTCR[a]		GFAPβgal × βgalTCR[b]		
Immunization	incidence	severity	incidence	severity	P value
βgal + PTx	1/15	(1.0, 2.5)	11/18	1.0, 1.0, 1.0, 1.5,1.5, 1.5, 1.5, 1.0,1.0, 1.5, 2.0	0.0019[c]
PTx only	ND	ND	0/5	0	0.007[d]

[a] EAU 35 days post-immunization.

[b] brain inflammation 21 days post-immunization.

[c] Fisher's exact test; 2-tailed; arrβgal × βgalTCR vs. GFAPβgal × βgalTCR.

[d] Fisher's exact test; 2-tailed; GFAPβgal × βgalTCR mice; βgal + PTx vs. PTx only incidence.

Table V

Analysis of T cells from Rag$^{(-/-)}$ and arrβgal × Rag$^{(-/-)}$ mice transferred with CD4$^+$CD25$^-$βgalTCR T cells

		T cell Recovery				
Recipient Mice	**N**	**number of CD4$^+$Vβ10$^+$ (x 10^6)[a]**	**number of CD4$^+$Vβ10$^+$ CD25$^+$ (x 10^4) [a]**	**number of CD4$^+$Vβ10$^+$ Foxp3$^+$ (x 10^4) [a]**	**number of CD4$^+$Vβ10$^+$ CD25$^+$ Foxp3$^+$ (x 10^4) [a]**	**CD4$^+$Vβ10$^+$ % T$_{regs}$**
Rag$^{(-/-)}$	12	1.06 ± 0.69	17.60 ± 9.4	5.21 ± 3.94	4.77 ± 3.86	4.29 ± 3.27
arrβgal × Rag$^{(-/-)}$	11	0.59 ± 0.24	7.72 ± 2.28	2.03 ± 1.55	1.79 ± 1.53	3.75 ± 3.08
P value[b]		0.0414	0.0024	0.0206	0.0267	0.6870

[a] Mean of total cells per animal ± standard deviation ten weeks post-transfer.

[b] Student's t test, two-tailed.