# Induction of regulatory T cells by the immunomodulating cytokines α-melanocyte-stimulating hormone and transforming growth factor-β2

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**Abstract: Recently, we have reported that the cytokines α-melanocyte-stimulating hormone (α-MSH) and transforming growth factor-β2 (TGF-β2) work in synergy to induce the activation of regulatory T (Treg) cells. When we used α-MSH and TGF-β2 to generate ocular autoantigen-specific Treg cells and adoptively transferred them into mice susceptible to experimental autoimmune uveoretinitis (EAU), there was suppression in the incidence and severity of EAU. Specificity to a retinal autoantigen was required for the Treg cells to suppress EAU. When stimulated, these Treg cells produced TGF-β1, and their production of interferon-γ, interleukin (IL)-10, and IL-4 was suppressed. Also, the Treg cells are suppressed in their proliferative response. Our results demonstrate that α-MSH with TGF-β2 induce Treg cells that can subdue a tissue-specific autoimmune response. This also promotes the possibility of using these immunomodulating cytokines to purposely induce antigen-specific Treg cells to prevent and suppress autoimmune disease. *J. Leukoc. Biol.* 72: 946–952; 2002.**

***Key Words:*** *regulatory T lymphocytes · regional immunity · immunosuppression*

## INTRODUCTION

We have recently reported that α-melanocyte-stimulating hormone (α-MSH) mediates the induction of regulatory $CD25^+$ $CD4^+$ T cells (Treg cells). In addition we have reported that transforming growth factor-β2 (TGF-β2) but not TGF-β1 enhances α-MSH induction of Treg cells [1–3]. The induction of Treg cells by α-MSH requires the T cells to be memory or primed T cells, to be exposed to α-MSH when they are activated through their T cell receptor, and to express the melanocortin 5 receptor [2, 4]. These Treg cells are antigen-specific and require the expression of their specific antigen in a target tissue to mediate immunosuppression [2]. However, the Treg cells are able to suppress the activation of neighboring T cells responding to other antigens.

The neuropeptide α-MSH is a 13 amino acid-long protein produced by a limited number of cells, and like most neuropeptides, α-MSH and its receptors are evolutionarily conserved [5–8]. Originally described as an inducer of melanogenesis in frogs [9], in mammals, α-MSH is a potent suppressor of bacterial endotoxin-mediated inflammation and inflammatory activity of macrophages and neutrophils induced by interleukin (IL)-1, tumor necrosis factor α, and interferon-γ (IFN-γ) [10–13]. The anti-inflammatory activities of α-MSH are important in regulating a normal, inflammatory response [14]. We have found that this neuropeptide also regulates adaptive immunity, suppressing the induction of delayed type hypersensitivity (DTH) and autoimmune disease [2, 4, 15, 16]. At its physiological concentration (30 pg/ml), α-MSH suppresses IFN-γ production by activated, primed T cells and enhances T cell production of TGF-β1 [2, 4, 15, 17]. We have further characterized the TGF-β-producing T cells to be Treg cells. We have used these α-MSH/TGF-β2-induced Treg cells to suppress adoptive transfer of DTH [2]. In that study, we reported that it is possible to generate antigen-specific Treg cells by α-MSH in vitro and adoptively transfer them intravenously (i.v.) to suppress antigen-specific T helper type 1 (Th1) cells mediating inflammation. In addition, we found that the Treg cells could suppress a DTH response mediated by Th1 cells specific to another antigen, as long as the antigen presenting cells (APC) also presented the specific antigen for the Treg cells in the target tissue.

As we had demonstrated that α-MSH/TGF-β2-induced Treg cells suppress DTH in vivo when adoptively transferred under antigen-regulated conditions, we therefore asked whether it is possible to generate the α-MSH/TGF-β2 Treg cells specific to an autoantigen and suppress an autoimmune disease where the autoantigen is one of the antigens of the disease. We generated Treg cells and demonstrated their ability to suppress in an antigen-specific manner the incidence and severity of experimental autoimmune uveoretinitis (EAU), a mouse model of human posterior uveitis (inflammation of the eye).

## MATERIALS AND METHODS

### Animals and reagents

We obtained 6- to 8-week-old B10.RIII female mice from Jackson Laboratories (Bar Harbor, ME). The animals were cared for and used with approval from our

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Institutional Animal Care and Use Committee. Human interphotoreceptor retinoid binding protein peptide ($IRBP_p$) 161–180 (SGIPYIISYLHPGNTIL-HVD) was synthesized by the Analytical Biotechnology Service (Boston, MA). We purchased complete Freund's adjuvant (CFA) and desiccated *Mycobacterium tuberculosis* H37RA from Difco Laboratories (Detroit, MI). The ovalbumin (OVA), and MPL + TDM synthetic adjuvant was purchased from Sigma Chemical Company (St. Louis, MO). Purified retinal S-Ag (arrestin) was a gift from Dr. H. Ohguro (Hirosaki University, Japan). We purchased the cytokine α-MSH from Peninsula Laboratories (Belmont, CA) and TGF-β2 from R&D Systems (Minneapolis, MN).

## Antigen-pulsed APC

Spleen cells from naive mice suspended in RPMI 1640 (BioWhittaker, Walkersville, MD) plus 5% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) were seeded $1 \times 10^6$ cells into the wells of a 96-well flat-bottom culture plate. The cultures were incubated for 90 min at 37°C in 5% $CO_2$. The wells were washed twice with media. Added to the wells of washed adherent cells was $IRBP_p$ (5 μg/ml), OVA (100 μg/ml), or S-Ag (100 μg/ml). The cultures were incubated for an additional 24 h. Before using the cultured cells as antigen-pulsed APC, they were washed twice with serum-free media [15]: RPMI-1640 supplemented with 10 μg/ml gentamicin (Sigma Chemical Co.), 0.01 M Hepes, 1 × non-essential amino acids (NEAA) mixture, 1 mM sodium pyruvate (BioWhittaker), 1 mg/ml bovine serum albumin (BSA), and 1/500 dilution of "ITS+" culture supplement (Collaborative Biochemical Products, Bedford, MA).

## Induction of Treg cell

Primed T cells were isolated from draining lymph nodes and treated with α-MSH and TGF-β2 to induce regulatory activity as we had done previously [2]. Draining lymph nodes were collected from mice immunized 7 days previously to 50 μg $IRBP_p$, 100 μg S-Ag, or 100 μg OVA emulsified in MPL + TDM adjuvant. The draining lymph nodes were strained through a nylon mesh and made into a single-cell suspension in serum-free media. We purified the T cells using a mouse T cell-enrichment column (R&D Systems). The purified T cells were added to the wells ($1\times10^6$ cells/well) of the antigen-pulsed APC with the immediate addition of α-MSH (30 pg/ml), with TGF-β2 (5 ng/ml) added 4 h later. The T cell cultures were incubated for 24, 48, or 72 h depending on the experiment. For adoptive-transfer experiments, the T cell cultures were incubated for 24 h. Lymphokine production was assayed on the supernatants of T cell cultures incubated for 48 h. Proliferation was assayed by adding $^3$H-thymidine (0.5 μCi/ml; NEN Life Science Products, Boston, MA) to the T cell cultures that were incubated for 48 h. The T cell cultures were then incubated for an additional 24 h. The incorporated $^3$H-thymidine was measured as counts per minute (CPM) by liquid scintillation.

## Analysis of lymphokine production by in vitro-stimulated, primed T cells

To assay for T cell lymphokine production, the supernatants of T cell cultures incubated for 48 h were assayed by sandwich enzyme-linked immunosorbent assay (ELISA) for IFN-γ, IL-4, or IL-10. The wells of a microtiter plate were coated with capturing monoclonal antibody (Pharmingen, San Diego, CA) specific to the assayed lymphokine and were incubated overnight at 4°C. The plate was washed and blocked with 0.01 M phosphate-buffered saline (PBS) plus 1% BSA (PBS-BSA) and was washed with PBS-BSA. The culture supernatants and dilutions of standard recombinant lymphokine were added to the wells, and the plate was incubated for 3 h at room temperature. The plate was washed with PBS-BSA, and biotinylated-detecting antibody (Pharmingen) specific to the assayed lymphokine was added to the wells. The assay plate was incubated for 1 h and washed. Streptavidin-β-galactosidase was added to the wells, incubated for 30 min at room temperature, and washed. Chlorophenyl-red-β-D-galactoside substrate solution was added to the wells. The optical density (OD) of the color change was read with a 570-nm filter on a standard ELISA plate-reader. The concentration of lymphokines in the supernatant was determined from a standard curve calculated from the OD versus the concentration of the standard lymphokine solutions.

The concentration of mouse TGF-β1 in the T cell cultures was measured by the standard Mv1Lu cell bioassay as described previously [3]. The T cell cultures were washed for 24 h after stimulation to remove any remaining TGF-β2. The T cells were resuspended in 200 μl serum-free media and placed back into culture. The cultures were then incubated for an additional 24 h. The culture supernatants were collected and treated with acid for 1 h and neutralized. The transiently acidified samples were diluted 1:8 in 0.5% FBS Eagle's minimal essential medium and were added to cultures of $1 \times 10^5$ Mv1Lu cells in a flat-bottom 96-well culture plate. The cultures were incubated for 24 h at 37°C, and $^3$H-thymidine (0.5 μCi/ml) was added for the last 4 h. The incorporated $^3$H-thymidine was measured by scintillation. The concentration of TGF-β in the supernatants was determined from a standard curve calculated with the CPM of the Mv1Lu cells relative to known amounts of pure TGF-β1. The isoform of TGF-β in the culture supernatant was determined by including specific neutralizing antibodies against TGF-β1 [chicken anti-TGF-β1 immunoglobulin (Ig)Y, R&D Systems] or TGF-β2 (rabbit anti-TGF-β2 IgG, R&D Systems) in the assay.

## Ribonuclease protection assay (RPA)

Nonadherent cells (over 95% cells were $CD3^+$ cells as determined by flow cytometry) were collected 24 h after the T cells were antigen-activated in the presence of α-MSH and TGF-β2 as described above. Total mRNA was purified from these cells by TRI reagent LS (Molecular Research Center, Cincinnati, OH) and DNase I (Ambion, Austin, TX). RPA were performed using a RPA III kit (Ambion) with biotinylated uridine antisense riboprobes. The riboprobes were generated from custom templates (Pharmingen) using MAXI script T7 kits (Ambion) with Biotin-16-UTP (Enzo, Germany). Total mRNA (5 μg) was hybridized with the biotinylated probes overnight at 56°C. Unhybridized RNA was digested by RNase treatment. The remaining undigested (hybridized) RNA was loaded onto a 6% TBE/urea gel (Novex, San Diego, CA), run at 180 volts for 75 min. The electrophoresed RNA was electroblotted onto a Nylon membrane (Novex) and UV cross-linked. The membrane was blocked, incubated with streptavidin-alkaline phosphatase, and chemiluminescent substrate (BrightStar BioDetect, Ambion). The membrane was exposed to X-ray film, and the film was developed. The bands detected on a digitized image of the X-ray film were quantified by densitometric analysis (Image; National Institutes of Health, Bethesda, MD) and normalized to the L32 band.

## EAU and adoptive transfer of Treg cells

The B10.RIII mice were made susceptible to EAU by injecting 50 μg $IRBP_p$ emulsified with CFA containing 3.0 mg/ml *M. tuberculosis* H37RA into their footpad, base of tail, and back, subcutaneously [18]. On the same day of the immunization, mice were i.v. injected with $2 \times 10^5$ Treg cells from the 24-h cultures prepared above. The uveoretinitis was clinically assessed every 3 days by ocular fundus examination. Before the fundus examination, the pupils were dilated with 0.5% Tropicamide and 2.5% phenylephrine. The severity of inflammation was clinically graded on a scale of 0–5 (**Table 1**).

## Statistics

The difference between two mean clinical scores was statistically analyzed by the nonparametric Mann-Whitney test for the comparison of two independent populations. Differences in the incidence of EAU were statistically analyzed by

TABLE 1. The Criteria and Clinical Scoring for EAU

| Score | Criteria | | |
|---|---|---|---|
| 0 | No change | | |
| 1 | Mild vasculitis; <5 focal lesions | or | Soft exudates <5 spots |
| 2 | Severe vasculitis (thick wall, linear lesion) <half of retina | or | Multiple soft exudates <half of retina |
| 3 | Severe vasculitis (thick wall, linear lesion) ≥half of retina | or | Multiple soft exudates ≥half of retina |
| 4 | Retinal hemorrhages | or | Severe soft exudates |
| 5 | Subretinal and vitreous hemorrhages | or | Retinal detachment |

the Fisher exact test. Differences were called significant when the $P$ value of a statistical analysis was equal to or less than 0.05.

## RESULTS

### Treg cells specific for ocular autoantigens suppress autoimmune uveitis

In the B10.RIII mice, EAU is an autoimmune, T cell-dependent inflammatory disease of the retina with maximum uveoretinitis about 15 days after an immunization with $IRBP_p$ and CFA [19]. The disease is limited to 30 days, and clinical uveoretinitis is no longer detectable after 35 days.

By following our previous procedures [2], we treated primed T cells with α-MSH and TGF-β2, and they were restimulated in vitro with $IRBP_p$-pulsed APC. We use these cells as Treg cells in the following experiments. We i.v. injected these Treg cells ($2\times10^5$ cells/mouse) into mice susceptible to EAU. Every 3 days, we examined and scored the ocular fundus (retina and choroid). A mouse was considered to have uveoretinitis if at least one of its eyes had a score of two or more. It was possible to obtain a score of one with only an adjuvant injection but no higher without $IRBP_p$ antigen in the immunization. The severity of uveoretinitis is represented as the highest clinical score achieved by either eye in a mouse over the 25 days of the clinical disease (**Fig. 1**). Usually the maximum clinical uveitis scores of both eyes were the same.

Mice injected with the Treg cells significantly lowered the maximum scores of uveoretinitis and lowered the incidence of disease (Fig. 1). EAU-susceptible mice not injected with T cells had an 84% incidence of uveoretinitis and a mean maximum EAU score of 2.9 ± 1.2 (Fig. 1). The EAU-susceptible mice that were injected with the $IRBP_p$-specific Treg cells had a 44% incidence of uveoretinitis and a mean maximum EAU score of 1.4 ± 1.3 (Fig. 1). EAU-susceptible mice injected with naive T cells had no change in their incidence or severity of uveoretinitis, whereas there was 100% incidence of uveoretinitis with a mean maximum EAU score of 4.0 ± 0.0 (data not shown) in EAU-susceptible mice injected with untreated $IRBP_p$-specific T cells. Therefore, α-MSH and TGF-β2 mediated the induction of ocular autoantigen-reactive Treg cells that can function in vivo to suppress autoimmune uveoretinitis.

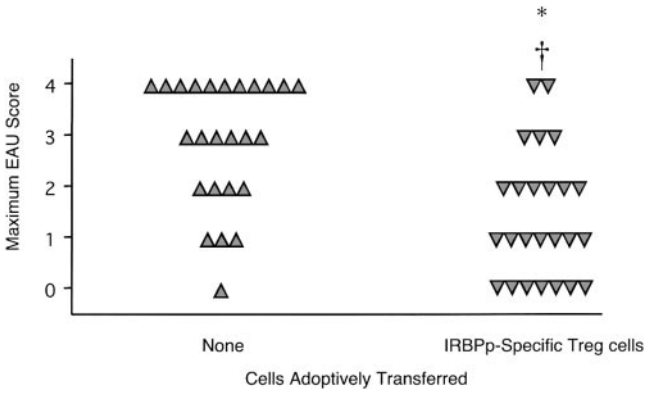


**Fig. 1.** α-MSH/TGF-β2-induced activation of Treg cells specific to $IRBP_p$ suppress EAU. EAU-susceptible B10.RIII mice were i.v. injected with $2.5\times10^5$ $IRBP_p$-specific Treg cells (downward triangles) induced in vitro by α-MSH and TGF-β2 as described in Materials and Methods. Control EAU mice were not injected with cells (upward triangles). The severity of inflammation was clinically graded on a score of 0–5 (see Table 1) from ocular fundus examinations of the mice every 3 days. The data presented are the maximum clinical score obtained by each mouse. Also presented is the ratio of mice with EAU to total mice tested. *, The clinical scores are significantly ($P\leq0.05$) different between the two groups of mice. †, The incidence of EAU is significantly ($P\leq0.004$) different between the two groups of mice.

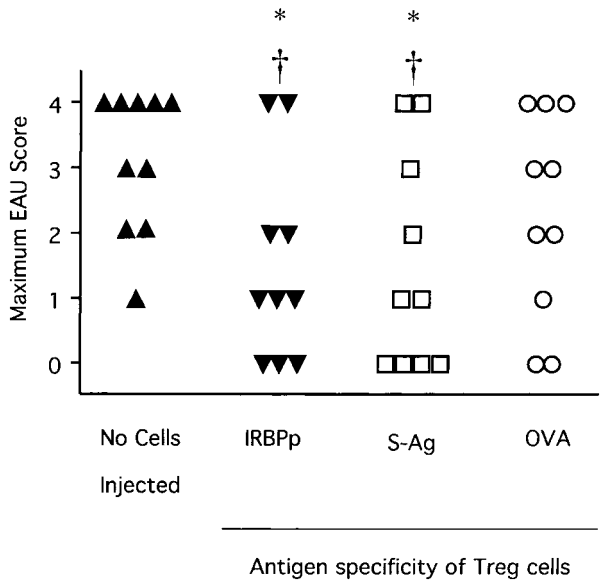


**Fig. 2.** The α-MSH/TGF-β2-induced Treg cell suppression of EAU is ocular autoantigen-specific. EAU-susceptible mice were injected with $2.5\times10^5$ $IRBP_p$, S-Ag, or OVA-specific Treg cells. The uveoretinitis was scored, and presented is the maximum clinical score obtained by each mouse. Also presented is the ratio of mice with EAU to total mice tested. *, The clinical scores of these groups are significantly ($P\leq0.05$) different from the clinical scores of EAU-susceptible mice not injected with Treg cells. †, The incidence of EAU is significantly ($P\leq 0.03$) different from the incidence of EAU in mice not injected with Treg cells.

### Treg cells require specificity to an ocular antigen to suppress EAU

Previously, we had found that our α-MSH/TGF-β2-induced Treg cells were antigen-specific [1, 2]; therefore, we ask whether this requirement is also needed for our adoptively transferred Treg cells to suppress EAU (**Fig. 2**). We generated α-MSH/TGF-β2-induced Treg cells specific to $IRBP_p$, the antigen used to induce the autoimmune disease, to S-Ag (arrestin), another ocular-restricted autoantigen, or to OVA, an irrelevant antigen. The adoptive transfer of $IRBP_p$ or S-Ag-specific Treg cells significantly lowered the maximum scores of uveoretinitis and lowered the incidence of disease (Fig. 2). The OVA-specific Treg cells, which we have previously shown are extremely effective in suppressing an OVA-specific DTH [2], had no affect on the severity or incidence of the disease (Fig. 2). These results demonstrate that for the α-MSH/TGF-β2-induced Treg cells to suppress autoimmune uveoretinitis, they must be specific to a retinal autoantigen, but it is not necessary for the specificity to be against the antigen used to initiate the disease.

## Characteristics of α-MSH and TGF-β2-treated effector T cells

We assayed the culture supernatants of our functioning α-MSH/TGF-β2-induced Treg cells specific to $IRBP_p$ for characteristic lymphokines and proliferation. In comparison to the cultures of the same primed T cells not treated with α-MSH and TGF-β2, the Treg cell cultures were significantly suppressed in IFN-γ, IL-10, and proliferation (**Fig. 3**). We could not find IL-4 in any of the cultures above the background of the assay (data not shown). As IL-10 and TGF-β1 can be produced by the APC in the cultures, we assayed the supernatants of APC cultured without the T cells for IL-10 and TGF-β1 (Fig. 3e). We found that untreated APC produced IL-10 at a concentration we found in the untreated T cell cultures and that α-MSH and TGF-β2 treatment suppressed the IL-10 production (Fig. 3, c and e). In addition, APC treated with α-MSH and TGF-β2 produced TGF-β1 at 20% of the TGF-β1 concentration found in the T cell cultures (Fig. 3, b and e). Therefore, all of the IL-10 in the T cell cultures are from the APC, and most (80%) of the TGF-β1 is from the treated T cells. The antigen activation of primed T cells in the presence of α-MSH and TGF-β2 suppressed proliferation (Fig. 3d); however, we could not detect any change in cell viability between the treated and untreated T cells (data not shown). The antigen activation of primed T cells in the presence of α-MSH and TGF-β2 suppresses production of type 1 and type 2 lymphokines in favor of a lymphokine profile centered on the production of TGF-β1.

Along with assaying the culture supernatant for lymphokine protein, we isolated mRNA from the treated T cells and analyzed the mRNA by ribonuclease protection assay for IL-4, IL-10, IFN-γ, TGF-β1, and L32 mRNA (**Fig. 4**). The Treg cells demonstrate a significant reduction in the expression of IFN-γ RNA; however, their expression of TGF-β1 RNA did not significantly increase (Fig. 4) in proportion with the production of TGF-β1 protein (Fig. 3b). As we expected, there was no detectable IL-10 RNA in the untreated T cells (Fig. 4), as we know that it is the APC producing IL-10 in the cultures (Fig.

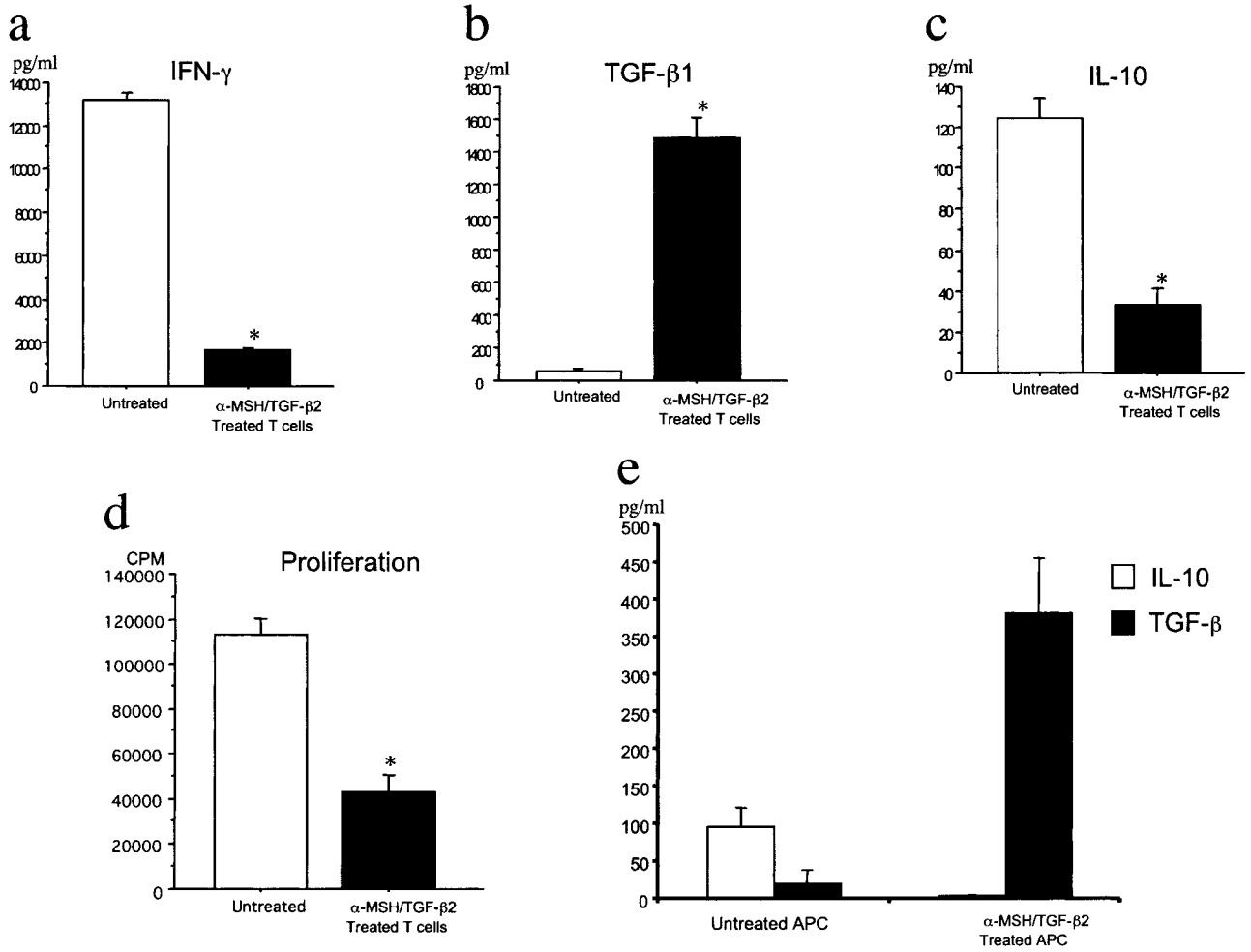


**Fig. 3.** α-MSH and TGF-β2 mediate differential lymphokine production by effector T cells. The $IRBP_p$-primed T cells ($1\times10^6$ cells) were activated in culture by $IRBP_p$-pulsed APC in the presence of α-MSH (30 pg/ml) and TGF-β2 (5 ng/ml) as described in Materials and Methods. The culture supernatants were assayed for cytokines (a–c) after 48 h of incubation. Proliferation (d) was assayed after 72 h of incubation. The concentration of IFN-γ (a) and IL-10 (c) was measured by specific sandwich ELISA and for TGF-β1 (b), by bioassay. The assay results are presented as the mean pg/ml ± SEM of four independent experiments. Proliferation (d) was detected by scintillation counting of incorporated $^3$H-thymidine and presented as CPM. (e) The production of IL-10 and TGF-β1 produced by the APC without T cells was also assayed. *, The mean is significantly ($P\leq0.05$) different from the untreated cells.

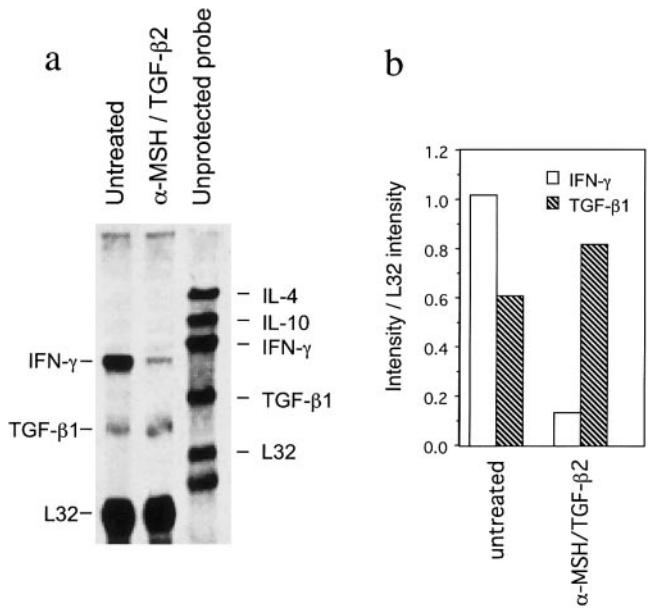


**Fig. 4.** α-MSH and TGF-β2 treatment changes the expression of IFN-γ and TGF-β1 mRNA in activated T cells. The $IRBP_p$-primed T cells were activated in culture by $IRBP_p$-pulsed APC in the presence of α-MSH and TGF-β2 as in Figure 3. After the cells were cultured for 24 h, the cultured T cells were isolated, and their mRNA was harvested and analyzed in a chemiluminescent-ribonuclease protection assay (a) using IL-4, IL-10, IFN-γ, TGF-β1, and L32 probes. The intensity (b) of the IFN-γ and TGF-β1 bands in each lane was quantified by densitometry and normalized to the band intensity of the housekeeping gene L32. The results presented are from one representative experiment of three with the same results.

3e) and not the T cells whose RNA we assayed in the ribonuclease protection assay. Therefore, the α-MSH/TGF-β2-induced Treg cells produce TGF-β1 without IL-4, IL-10, and IFN-γ. This lymphokine profile differentiates our Treg cells from the IFN-γ-producing Th1 cells and the IL-4, IL-10-producing Th2 cells.

## DISCUSSION

In this manuscript, we generated Treg cells specific for $IRBP_p$ by antigen-activated, primed T cells in the presence of α-MSH and TGF-β2. We demonstrated that when these Treg cells were adoptively transferred into EAU-susceptible mice, they suppressed the severity and incidence of EAU. In addition, we were able to suppress EAU by adoptively transferring α-MSH/TGF-β2-induced Treg cells specific to S-Ag, another ocular autoantigen. However, the adoptive transfer of OVA-specific Treg cells did not suppress EAU. Therefore, as predicted from our previous findings, there is a requirement for the α-MSH/TGF-β2-induced Treg cells to be specific to an antigen in the target tissue for the activation of their suppressive activity. It was not necessary for the Treg cell antigen specificity to be against the initial antigen target of the autoimmune disease. We cannot tell from these experiments whether the Treg cells suppressed EAU in response to antigens presented in the ocular microenvironment or prevented the presentation of other autoantigens associated with the usual antigenic spread of autoimmune diseases. However, the results do suggest that to suppress an autoimmune disease, we need to have the α-MSH/TGF-β2-induced Treg cells respond to an antigen in the target tissue.

Treatment of primed T cells with α-MSH and TGF-β2 suppressed the production of IFN-γ but enhanced TGF-β1 production. The T cells were also suppressed in proliferation. Previously, we have demonstrated that the production of TGF-β1 is important in the mechanism of suppression by the α-MSH/TGF-β2-induced Treg cells [2]. Our findings demonstrate that the α-MSH and TGF-β2 treatment suppressed transcription of IFN-γ RNA but not for TGF-β1. The enhanced production of TGF-β1 protein with no change in TGF-β1 RNA suggests a post-transcriptional regulation of TGF-β1 production. Such post-transcriptional regulation of TGF-β production is not uncommon [20, 21]. The result of treating the primed T cells with α-MSH and TGF-β2 is the induction of an effector T cell that produces TGF-β. This phenotype is stable, and the T cells function as regulatory T cells to suppress autoimmune disease in an antigen-specific manner.

It is clear that we have induced a population of T cells that are different from the accepted definitions of Th1 and Th2 cells. These cells function as Treg cells. The literature describes two types of $CD4^+$ Treg cells (reviewed in refs. [22, 23]). There is a natural population of thymus-derived $CD4^+$ Treg cells. These Treg cells are characterized as naive and suppress other T cells through a contact-dependent mechanism [24–27]. They stably express the IL-2-α receptor CD25, and although they require T cell receptor stimulation for survival and expansion, they are not dependent on antigen specificity for function. Th3 cells are another type of $CD4^+$ Treg cells induced by immunizing through specific tissues, such as through the mucosa in oral tolerance induction [28–32]. These Th3 cells emerge from a differentiated, primed T cell response. They are characterized by the production of TGF-β1 with sometimes IL-4 and IL-10 [29, 32]. They require antigen-specific stimulation to induce their suppressive activity, but they suppress other T cells through nonspecific mechanisms, presumably mediated by lymphokines. Our previous work has demonstrated that the α-MSH/TGF-β2-induced regulatory T cells are antigen-specific in their immunosuppressive activity [2]. We also know from our study of α-MSH-induced Treg cells that the Treg cells are $CD25^+$ $CD4^+$ and that neutralization of the TGF-β produced prevents their ability to suppress the activation of other T cells [4]. Therefore, our α-MSH/TGF-β2-induced Treg cells have the characteristics of Th3 cells, but we have no evidence to rule out the possibility that our α-MSH/TGF-β2 treatment promoted the activation of the naturally occurring $CD25^+$ $CD4^+$ Treg cells with antigen specificity. In either possibility, our treatment with α-MSH and TGF-β2 has converted a population of primed T cells that would mediate a severe autoimmune disease into a population of T cells that now suppresses the induction of the autoimmune disease.

The immunomodulating activity of α-MSH and TGF-β2 was originally defined by their constitutive, immunosuppressive activity in immune-privileged tissues [15, 17, 33–35]. Immune-privileged tissues are sites of extreme regional immunity. Our findings of an α-MSH and TGF-β2-induced Treg cell have important implications on the potential outcomes of an

immune response within an immune-privileged tissue. As a result of the constitutive expression of α-MSH and TGF-β2, the immune-privileged tissue microenvironment has the ability to manipulate immunity to further support and contribute to the normal immunosuppressive microenvironment through the activation of TGF-β-producing T cells [3]. As the normal ocular anterior chamber is an immune-privileged tissue [36], it is possible that T cells entering the normal ocular microenvironment are immediately influenced by the constitutively expressed α-MSH and TGF-β2. If the T cells are activated in the microenvironment, they could be converted into a TGF-β1-producing T cell. It is clear that under normal circumstances, Th1 cells are suppressed in the eye's anterior chamber [37]. Rats recovering from EAU have in their spleens $CD4^+$ Treg cells specific to ocular autoantigens [38]. Therefore, the ocular microenvironment retains, even in the presence of inflammation, the potential to mediate peripheral tolerance, which in turn could regulate and prevent reoccurrence of autoimmune disease. This may be more so for rodent models of autoimmune uveitis, as they do not suffer from spontaneous relapsing autoimmune uveitis. We have already demonstrated that α-MSH suppresses the inflammation associated with autoimmune disease [4]. Therefore, there is a possibility that relapsing or worsening of an autoimmune disease in the eye may be related to an inability to maintain or activate the Treg cells.

From our study of the mechanisms of ocular immune privilege, we have characterized the role of cytokines in regulating effector T cell activation and functionality. There is a potential for the ocular factors α-MSH and TGF-β2 to work in synergy to induce the activation of Treg cells [2]. This lesson of immune privilege has opened an opportunity to generate Treg cells against specific tissue autoantigens and to manipulate immunity to prevent the induction of deleterious immune responses. It has also led us to purposely induce tissue antigen-specific Treg cells to prevent and suppress autoimmune disease.

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