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Cytokine gene transduced T cells in the treatment of allergic encephalomyelitis and airway hypersensitivity

Research Article

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Abbreviations: encephalomyelitis, (EAE); expiratory time, (Te); inflammatory bowel disease, (IBD); Keyhole limpet hemocyanin, (KLH); latency associated protein, (LAP); myelin basic protein, (MBP); ovalbumin, (OVA); peak expiratory flow, (PEF); plasminogen activator inhibitor-1, (PAI-1); proteolipid protein, (PLP); relaxation time, (RT); spleen, (spl); *Staphylococcus* enterotoxin B, (SEB)

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Summary

TGF-β1 or IL-10 transduced myelin basic protein (MBP)-specific BALB/c cloned Th1 cells were injected into SJL x BALB/c F1 mice 11-15 days after immunization with proteolipid protein to induce EAE. TGF-β1/MBP T cells significantly ameliorated the EAE, while IL-10/MBP T cells were less effective. TGF-β1 transduced ovalbumin (OVA)-specific Th1 clones did not influence EAE, even when re-activated by OVA *in vivo*. However, TGF-β1/OVA T cells did protect against OVA-specific Th2-cell mediated airway hyper-reactivity induced by inhaled OVA. TGF-β1/KLH T cells did not prevent OVA-induced airway hyper-reactivity in mice sensitized and challenged with OVA alone, but did protect mice challenged with KLH + OVA. Thus, the antigen specificity of the Th1 cells allows site-specific delivery of therapeutic TGF-β1 to both Th1 and Th2 cell-mediated inflammatory infiltrates. EAE relapses, induced by bacterial superantigen or endotoxin within 2 weeks, but not >6 weeks, after transfer of TGF-β1 or IL-10/MBP T cells, were reduced. Relapses induced 5 weeks after immunization with PLP could be prevented by simultaneously injected TGF-β1/MBP cells. Spinal cords taken 12-50 days after TGF-β1/MBP cells contained TGF-β1 cDNA. Spinal cords from the majority of mice receiving IL-10/MBP cells contained IL-10 cDNA up to 2 weeks, but not 50 days after cell transfer. Thus, TGF-β1-transduced T cells may be useful in the therapy of autoimmune and allergic inflammatory diseases, but in the EAE model, the same approach with IL-10-transduced T cells appears less effective.

I. Introduction

Resistance to the induction of experimental autoimmune diseases, such as allergic encephalomyelitis (EAE), inflammatory bowel disease (IBD) and collagen induced arthritis (CIA), is often attributed to the presence of immune-regulatory T cells. Such T cells may either be induced prior to induction of the experimental autoimmune disorder by mucosal or systemic exposure to auto-antigens (Karpus and Swanborg, 1991; Khoury et al, 1992), or may be present spontaneously and expanded during the course of the disease. In the latter case, spontaneous recovery from an initial disease episode and subsequent resistance to reinduction of the disease is

attributed to the expansion of these immune-regulatory T cells (Ellerman et al, 1988; Kumar and Sercarz, 1993). One of the mechanisms by which such T cells are thought to curb inflammatory lesions characteristic of these autoimmune diseases is by producing and inducing anti-inflammatory cytokines, among which TGF-β, IL-10 and IL-4 have been implicated as very important. Indeed, under certain conditions, neutralization of these cytokines aggravates autoimmune diseases and/or interferes with the activity of immune-regulatory T cells (Kuruvilla et al, 1991; Racke et al, 1992; Johns and Sriram, 1993; Santambrogio et al, 1993; Santos et al, 1994; Stevens et al, 1994; Crisi et al, 1995, 1996; Powrie et al, 1996; Burkhart et al, 1999; Stohlman et al, 1999).

Administration of TGF-β, IL-10 or IL-4, however, only partially protects against autoimmunity. Treatment with active TGF-β is most effective when given during the latter part of the induction phase of EAE (Santambrogio et al, 1993) or CIA (Thorbecke et al, 1992), or at the time of passive induction of EAE with myelin protein-sensitized T cells (Racke et al, 1991; Stevens et al, 1994). This cytokine can also prevent the occurrence of relapses from EAE (Racke et al, 1993; Santambrogio et al, 1993). However, TGF-β cannot cause recovery from EAE or CIA, once the disease has developed. It is of interest that TGF-β1$^{-/-}$ and TGF-βRII$^{-/-}$ mice exhibit generalized and fatal T lymphocyte infiltrations in various organs (Diebold et al, 1995; Gorelik and Flavell, 2000). This indicates that TGF-β1 is a cytokine with significant anti-inflammatory and immunosuppressive properties, a key regulator in the maintenance of immunological homeostasis.

Injections of IL-4 or IL-10 are even less effective in modulating autoimmune diseases. These cytokines are reported to have either no effect or to offer protection only when administered early during disease induction (Rott et al, 1994; Santambrogio et al, 1995; Cannella et al, 1996). However, IL-10 knockout (IL-10$^{-/-}$) mice are very susceptible to induction of EAE, developing a more severe and persistent form of EAE than do IL-4$^{-/-}$ or wild-type mice (Bettelli et al, 1998; Samoilova et al, 1998). Moreover, IL-10 transgenic mice are resistant (Bettelli et al, 1998; Cua et al, 1999), while IL-4 transgenic and wild type mice are equally susceptible to induction of the disease. Treatment with IL-10, particularly when administered via the nasal route early during the induction of EAE decreases the severity of the disease (Xiao et al, 1998), but no such effect is observed when administration of IL-10 is delayed until after the initial induction phase or when it is given with sensitized T-cells, at the time of adoptive transfer of EAE (Rott et al, 1994; Nagelkerken et al, 1997). Similarly, neutralization of IL-10 in IL-10 transgenic mice prior to immunization with myelin proteins is needed to completely abolish the resistance of IL-10 transgenic mice to EAE (Cua et al, 1999). Thus, it seems that IL-10 may prevent the sensitization of encephalitogenic T-cells, but that it cannot reverse T-cell sensitization and EAE symptoms. It has also been shown that the local administration of the cDNA encoding viral IL-10 into knee-joints of rabbits can reduce the inflammatory lesions provoked by the intra-articular injection of ovalbumin into ovalbumin pre-sensitized animals (Lechman et al, 1999).

T cells from multiple sclerosis patients reportedly produce less TGF-β1 in culture than do T cells from normal individuals (Mokhtarian et al, 1994). If TGF-β producing T cells are important in the curtailment of autoimmunity, as also suggested by the observations on TGF-β$^{-/-}$ mice (Diebold et al, 1995), treatment with auto-reactive T cells which have been engineered to produce excess TGF-β might be beneficial. To determine whether auto-reactive T cells which produce IL-10 or TGF-β1 are capable of down-regulating autoimmune disease, we have artificially increased the ability of myelin basic protein (MBP)-specific BALB/c cloned T cells to produce either IL-10 or latent TGF-β1 by transducing them with a recombinant retrovirus engineered to contain the cDNA for one of these cytokines. In previous studies (Chen et al, 1998), we showed that TGF-β1-transduced myelin basic protein (MBP)-specific T cells lose the capacity to provoke EAE in BALB/c mice, and gain instead the ability to protect against EAE, induced in (SJL x BALB/c) F1 mice by immunization with proteolipid protein (PLP). In similar studies on EAE with IL-4 transduced T hybridoma cells (Shaw et al, 1997) and IL-10 transduced T cells (Mathisen et al, 1997) protective effects were also reported. In the latter report, the transduced T cell clone also showed a high level of endogenous IL-10 production, and was not examined for production of other cytokines. It is therefore not certain whether or not the human IL-10, used for the transduction of these cells, was responsible for the protective effect against EAE.

Most of the autoimmune diseases for which a protective role for immunosuppressive cytokines, such as TGF-β and IL-10, has been described are Th1 cell mediated diseases. To determine whether typical Th2 cell induced inflammatory diseases, such as airway hyper-reactivity or asthma, are also down regulated by these cytokines, a similar approach was used in an animal model for asthma. TGF-β1-transduced OVA-specific Th1 cells were found to protect against OVA-specific Th2 cell-induced airway hyper-reactivity (Hansen et al, 2000). Thus, use is made of the migratory properties of antigen-specific activated cloned T cells to obtain enhanced local production of an immune-regulatory cytokine within inflammatory infiltrates, and thereby ameliorate the inflammation.

In the present study, a comparison was made of the relative effectiveness of IL-10 and TGF-β1 transduced MBP-specific T cells in protecting against EAE and relapses of EAE induced by bacterial superantigen or lipopolysaccharide. In addition, the requirement for antigen specificity in exerting protection was further examined for TGF-β1 transduced T cells in both the mouse model of asthma and in EAE.

Table 1

CYTOKINE PRODUCTION BY TRANSDUCED TH1 CELLS		
Cells Tested ----------	TGF-β1 (ng/ml)* ----------	IL-10 (ng/ml)** ----------
Untransduced MBP/Th1	0.037	< 0.03
TGF-β1 Transduced MBP/Th1	2.3	NT
IL-10 Transduced MBP/Th1	NT	3.0
TGF-β1 Transduced KLH/Th1	3.5	NT
TGF-β1 Transduced OVA/Th1	3.6	NT

* TGF-β1 content of media collected after 24 h of culture of 10^6 cloned T cells/ml. The medium was supplemented with 1% Nutridoma and contained no serum. ELISA was used to assay the TGF-β1 after activation with acid. No active TGF-β was detected when the activation step was omitted.

** IL-10 contents as assayed by ELISA on culture fluids from 10^6 cells/ml, incubated for 24 h in ISCOVE's medium with 10% fetal calf serum. The assay was performed a few times during the course of these experiments with similar results (range of 1.1-3 ng/ml), suggesting that the rate of IL-10 production did not vary significantly.

II. Results

A. Characterization of transduced T cell clones

Transduced T cell clones were identified by the presence of cDNA for latent TGF-β1 or IL-10 as determined by PCR. TGF-β1 or IL-10 transduced and untransduced T cell clones were then compared with respect to their ability to produce the relevant cytokine. Acid treated and untreated serum-free tissue culture medium from antigen activated and resting TGF-β transduced and control T cell clones (10^6 cells/ml, 24 h at 37°C) were analyzed for latent and active TGF-β1 contents, respectively. All three of the latent TGF-β1 transduced clones, MBP-, OVA-, and KLH-specific T cells, exhibited 1.5-4 ng/ml of latent TGF-β in their supernatants, whether or not they had been activated by antigen (**Table 1**). Supernatants from untransduced T cell clones showed barely detectable amounts of TGF-β1 above the serum-free medium background (< 0.1 ng). Serum containing supernatants from untransduced T cells contained <0.05 ng of IL-10 per ml, while the supernatants from IL-10 transduced T cells contained 1.05-3.0 ng/ml.

To determine whether the production of latent TGF-β1 caused a change in the cytokine pattern produced by the MBP-specific T cell clone, a ribonuclease protection assay was performed on RNA prepared from the untransduced and the TGF-β1-transduced MBP-specific T cell clone 2-3 days after activation of the cells by antigen, using two sets of cytokine probes. The mRNA distribution for LTα, LTβ, TNF-α, IFN-γ, TGF-β2, TGF-β3 and MIF, expressed as a percentage of mRNA for the housekeeping gene, GAPDH, was the same for the untransduced and transduced clones. However, transduction caused an increase in TGF-β1 mRNA. The results obtained with the other set of cytokine probes showed an absence of mRNA for IL-4, IL-5, IL-6 or IL-10 before and after transduction in the TGF-β1 transduced clone (Chen et al, 1998). The IL-10 transduced clone was examined similarly. Again, no difference in the representation of mRNA for any of the other cytokines was found, even after prolonged propagation of these IL-10 transduced Th1 cells, but a marked increase in the mRNA for IL-10 was seen (data not shown).

The transduced MBP-specific T cells were also characterized with respect to their ability to proliferate in response to antigen (MBP peptide 59-76) *in vitro*. For both TGF-β1/MBP and IL-10/MBP cells, the dilution of carboxy fluorescein diacetate (succinimidyl ester, CFSE (Lyons and Parish, 1994)) used as label was similar to that in control (untransduced) cells over a period of 3 days in culture after exposure to MBP, and the incorporation of ^{3}H-thymidine at the end of the 3-day culture period was also comparable to that in control cells (data not shown).

B. Comparisons of TGF-β and IL-10 transduced MBP specific T cells in vivo

In previous work we showed that TGF-β1 transduced MBP-specific T cells were able to ameliorate the course of actively induced EAE when transferred approximately at the time of first appearance of disease symptoms, i.e. 11-15 days after immunization with PLP in CFA. In order to compare the effects of IL-10/MBP and TGF-β1/MBP T cells, both transduced cells from the same original Th1 clone were activated *in vitro* by exposure to MBP and then injected into SJL x BALB/c mice, 11-13 days after the mice had been immunized with PLP in CFA. The results in **Figure 1A** show that there was an immediate effect of

the TGF-β1 transduced cells, such that the severity of EAE that had already developed in these recipients did not increase any further. In contrast, both groups of mice that either received no cells or IL-10 transduced T cells showed a marked increase in EAE severity until day 15. In this experiment there was no protective effect of the IL-10 transduced cells, but in a repeat of this experiment (**Figure 1B**), the severity of EAE in the mice receiving no cells remained higher between days 16 and 21 than in the mice receiving IL-10 transduced T cells, although this effect was not statistically significant. It should also be noted that untransduced T cells caused a significant increase in severity of EAE symptoms between days 14 and 16, which was not seen in recipients of IL-10 transduced cells, indicating that the augmented production of IL-10 in these cells prevented them from increasing the severity of the EAE.

It was possible that the IL-10/MBP T cells did not reverse EAE because they failed to enter the CNS and/or failed to proliferate locally. We, therefore, analyzed recipients' spinal cords and lymphoid tissue to determine whether cDNA for IL-10 could be detected. The results in **Figure 2** show that, indeed, IL-10 cDNA was detectable in the spinal cord of the majority of recipients killed during the first two weeks, but could no longer be detected 50 days after T cell transfer.

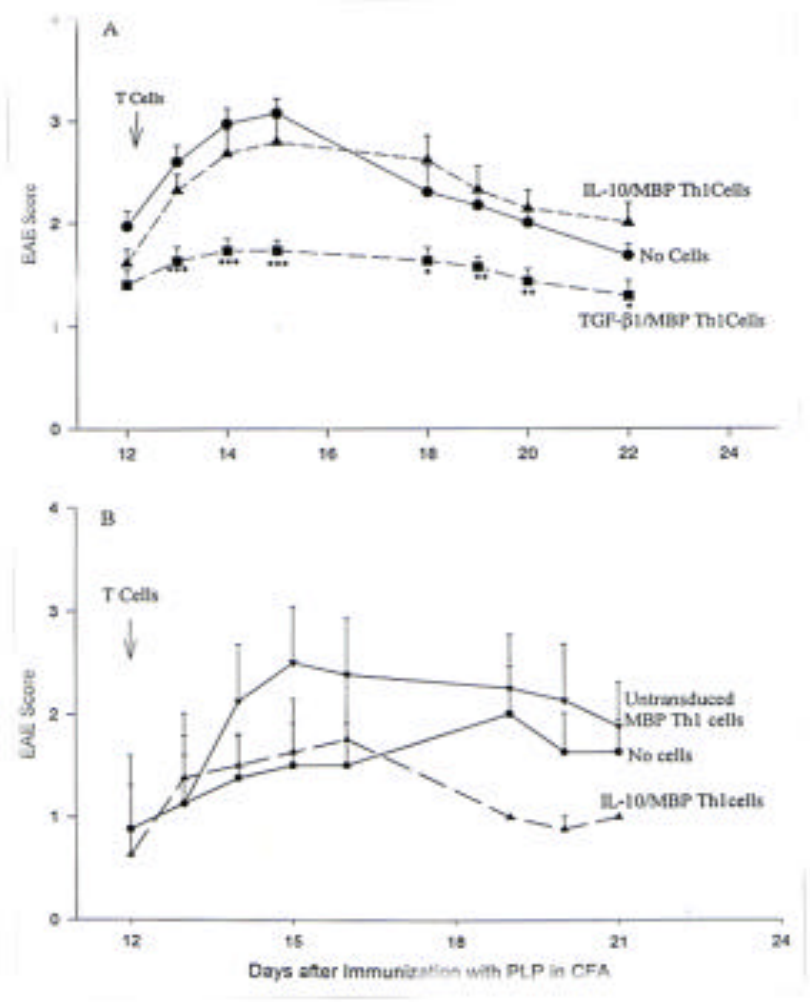

Figure 1. Effect of IL-10 and TGF-β transduced and untransduced MBP-specific cloned Th1 cells on EAE severity. SJL x BALB/c F_1 mice were immunized with PLP peptide (139-151) in CFA on day 0. On day 12, 3 x 10^6 Th1 cells were injected iv. **A)**: Comparison of IL-10 and TGF-β transduced cells. ●¾● No cell control (n=14); ▲---▲ IL-10/MBP Th1 (n=15); ■---■ TGF-β/MBP Th1 (n=15). Statistical significance (Student T test):* p<0.05; ** p<0.01; *** p<0.001. **B)**: Comparison of IL-10 transduced and untransduced cells. ●¾● No cell control, n=4; ▲---▲ IL-10/MBP Th1 (n=4); ▼---▼ Untransduced MBP Th1 cells (n=4).

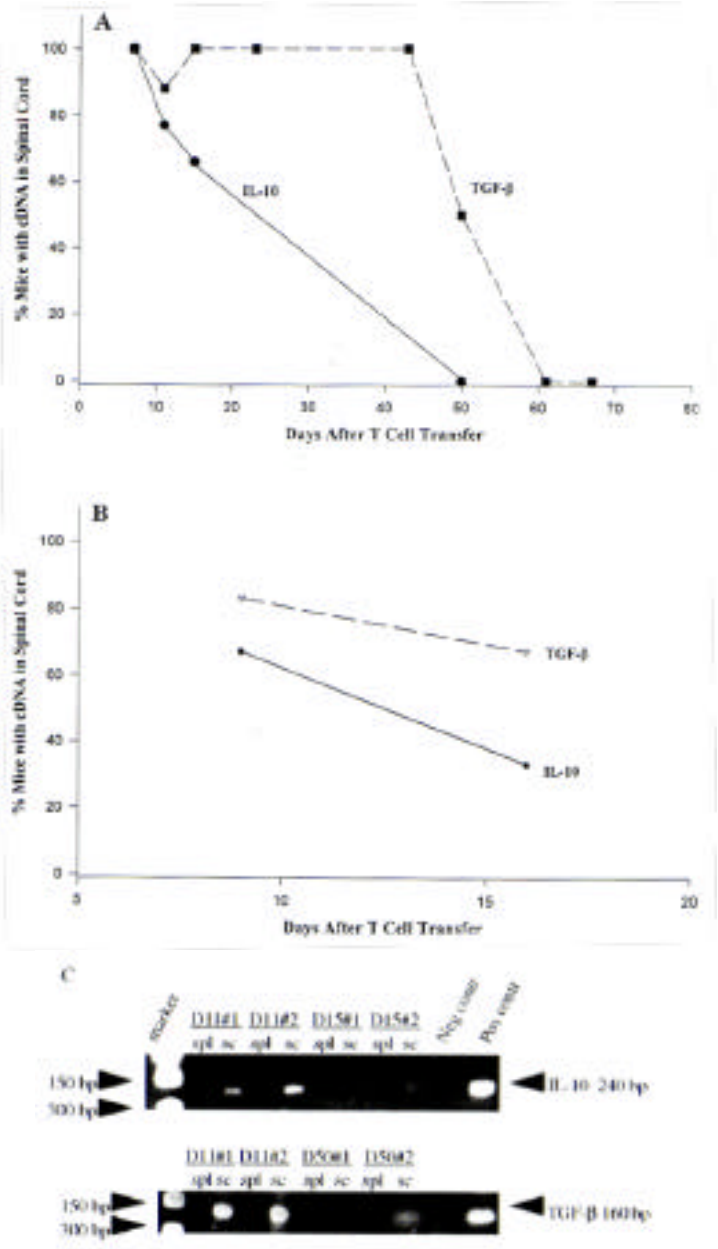

Figure 2. Detection of cDNA of TGF-β1 or IL-10 in spinal cords from mice receiving transduced Th1 cells after induction of EAE. Total DNA was extracted from the spinal cords of mice at different intervals after IL-10/MBP or TGF-β1/MBP T cells had been injected. PCR was used to identify the IL-10 or TGF-β cDNA in the total DNA from individual spinal cords. **A**) Percentage of mice in which cDNA could be detected in spinal cord at different intervals upon cell transfer on day 11-15 after induction of EAE. ●¾● IL-10/MBP T cells (n=18); ■---■ TGF-β1/MBP T cells (n=28). **B**) Percentage of mice in which cDNA could be detected in spinal cord at different intervals upon cell transfer on day 34 after induction of EAE (at time of LPS injection). ●¾● IL-10/MBP T cells (n=9); --- TGF-β1/MBP T cells (n=9). **C**) Typical PCR products found in DNA from spinal cord (sc), but not in DNA from spleen (spl) at various days after T cell transfer (D11, 15, 50).

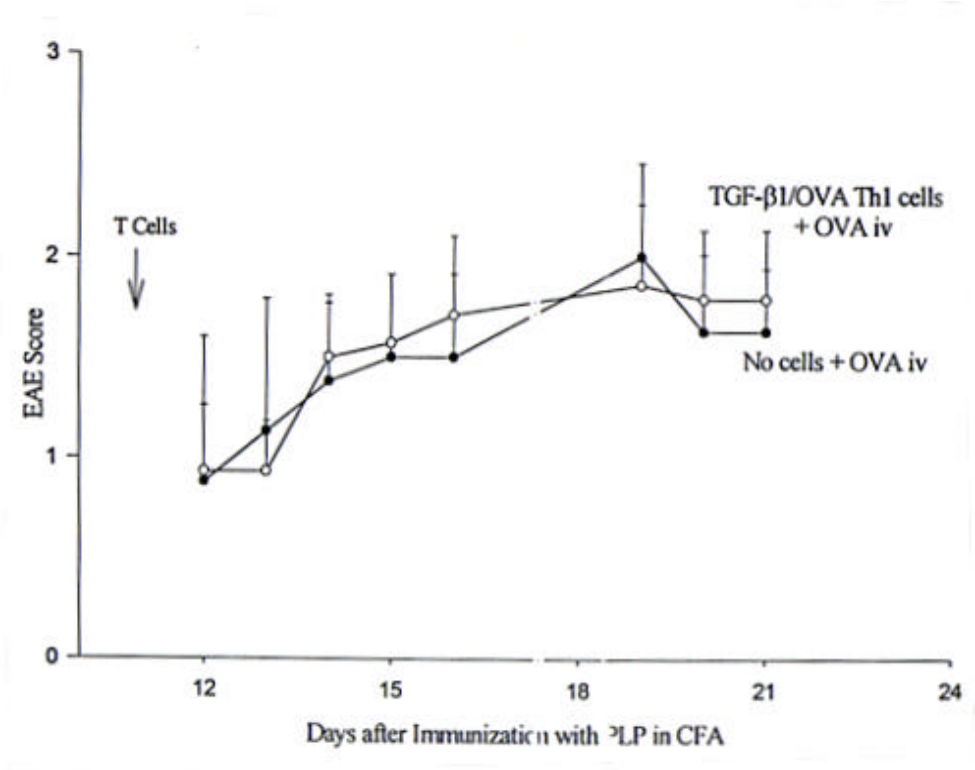

Figure 3. TGF-β transduced ovalbumin (OVA) specific T cells have no inhibitory effect on EAE development, even when the cells are reactivated in vivo by injection of OVA iv. O¾O TGF-β1/OVA Th1 cells on day 11, followed by iv injection of 50 μg of OVA (n=4); ●¾● Injection of 50 μg OVA alone on day 11 (n=4).

In contrast, the majority of mice receiving TGF-β1/MBP cells still had detectable cDNA for that cytokine in their spinal cords at 6 weeks after transfer. Thereafter, however, TGF-β1 cDNA also became undetectable. Neither cDNA was detectable in lymphoid tissue (spleen) either early or late after T cell transfer. Thus, a relatively effective accumulation of the transduced cells in the CNS occurred followed by their gradual disappearance.

C. Requirement for antigen specificity of TGF-β1 transduced T cells

We previously showed that, in order for TGF-β1 transduced T cells to have a protective effect against EAE development, they had to be specific for a myelin antigen (Chen et al, 1998). TGF-β1 transduced KLH or OVA specific Th1 cells did not have such an effect. Similarly, in the experiments on airway hyper-reactivity induced by OVA, TGF-β1/OVA cells protected but TGF-β1/KLH cells did not. In the present study, we analyzed this requirement for antigen specificity in more detail. In the experiments on EAE, in addition to exposing the T cells in vitro to the relevant antigen (OVA) a few days prior to transfer, we also injected the recipients on day 14 with 100 μg OVA ip to obtain additional activation of these cells in the mice. However, as can be seen from the results in **Figure 3**, there was no effect from these OVA specific T cells, whether the mice were injected with OVA or not (not shown).

Since the TGF-β1/MBP T cells enter the CNS, it is possible that the continued local stimulation in the spinal cord within local inflammatory lesions allows for activation of the latent TGF-β1 that they produce constitutively. However, in the EAE model, it is impossible to provide T cells of other specificities such as OVA or KLH with the antigen to which they respond locally within the CNS. The aspect of bystander effect was therefore further analyzed in the model of airway hyper-reactivity, where local exposure to any antigen can readily be performed by adding that antigen to the challenge inhalation. Indeed, when KLH was added to the OVA used for the challenge inhalation, TGF-β1/KLH Th1 cells could protect against airway hyper-reactivity in mice immunized to OVA (**Figure 4A**), although they were still less effective than TGF-β1/OVA Th1 cells (**Figure 4B**).

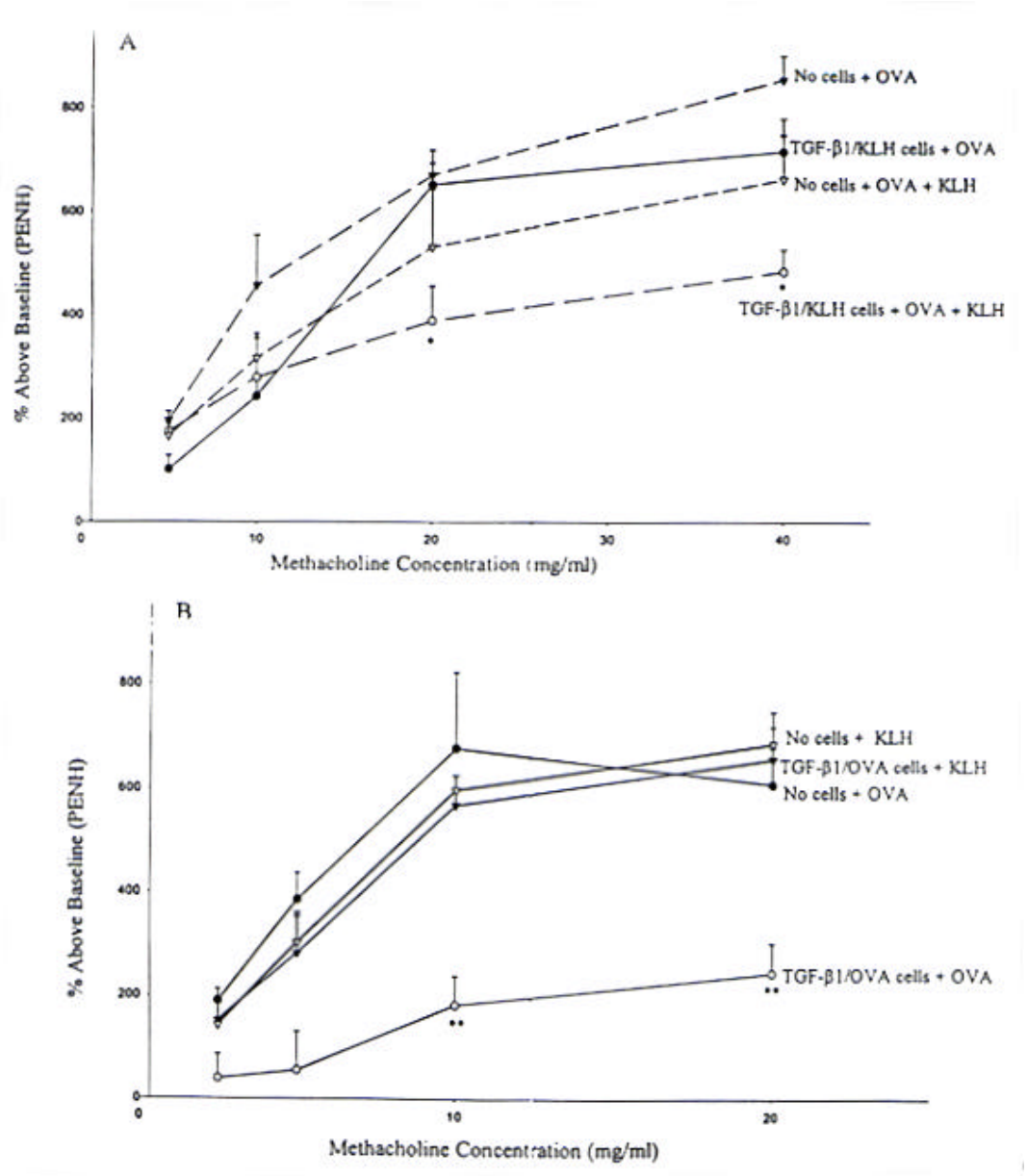

Figure 4. A protective effect of TGF-β1/KLH Th1 cells against OVA induced airway hyper-reactivity can be obtained if KLH is added to the challenge inhalation of antigen. BALB/c mice were immunized with OVA i.p. (50 μg) complexed with alum on day 1, and challenged intranasally on days 7, 8 and 9 with either 50 μg OVA alone or with 25 μg KLH + 50 μg OVA. **A**): ●¾● TGFβ1/KLH Th1 cells + OVA alone; ▼---▼ No cells + OVA; --- No cells + OVA and KLH; O---O TGFβ1/KLH Th1 cells + OVA and KLH, * p vs TGFβ1/KLH cells + OVA alone <0.05 (n=3); **B**): ¾ No cells + KLH; ●¾● No cells + OVA; ▼¾▼ TGF-β1/OVA Th1 cells + KLH; O¾O TGF-β1/OVA Th1 cells + OVA, ** p vs TGFβ1/OVA cells + KLH <0.01 (n = 3).

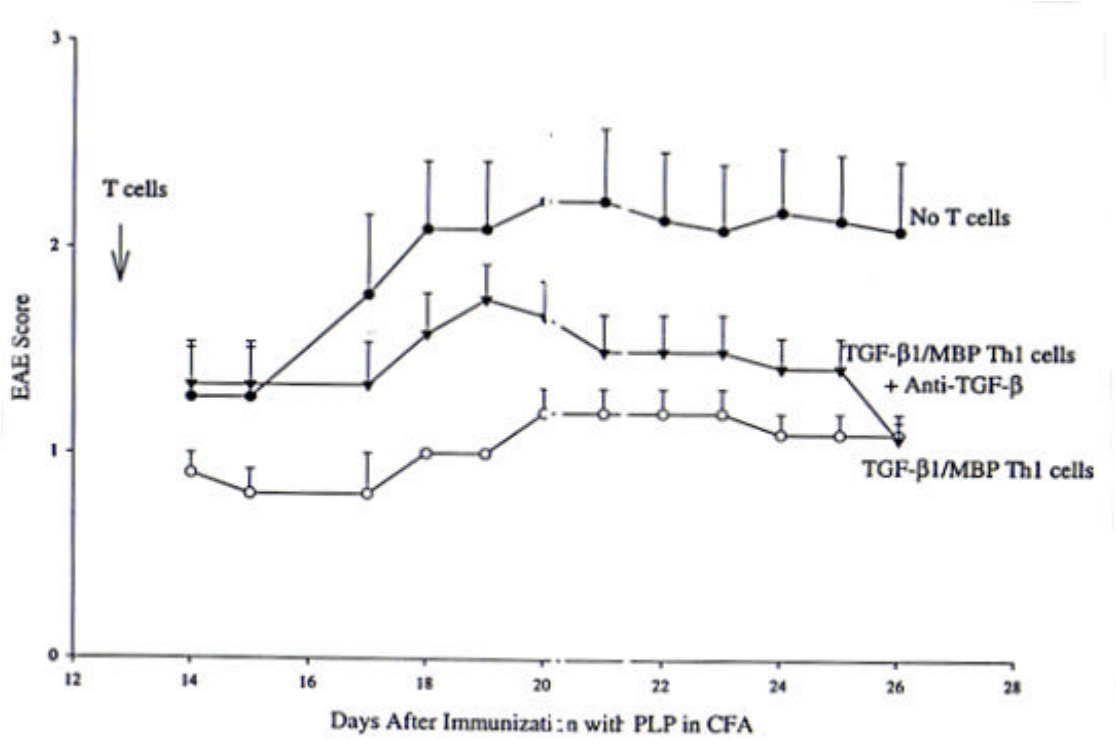

Figure 5: Anti-TGF-β mAb (2G7, 0.5 mg/mouse, ip), injected on the same day as the T cells (day 13) reduces the inhibitory effects of TGF-β MBP cells on EAE development. ●¾● No cells control (n=6); ▼¾▼ TGF-β 1/MBP Th1 cells with anti-TGF-β mAb (n=6); O¾O TGF-β 1/MBP Th1 cells alone (n=5).

Table 2

EFFECT OF TGF-β1/MBP T CELLS ON EAE RELAPSE INCIDENCE INDUCED BY SEB OR LPS			
Expt. #	TGF-β/MBP-Specific T Cells Injected	Relapse Induced With SEB or LPS	EAE Relapse Incidence*
1	Day 13 (after PLP in CFA)	SEB on Day 26	0/11
	None	SEB on Day 26	3/8
2	Day 12 (after PLP in CFA)	SEB on Day 58	5/10
	None	SEB on Day 58	11/14
2	Day 12 (after PLP in CFA)	LPS on Day 70	7/9
	None	LPS on Day 70	9/13

* Mice were considered to have relapsed when their disease incidence had increased by 0.5 or more for at least two consecutive readings within 3 days after injection of the SEB or LPS.

D. Influence of IL-10 and TGF-β1 transduced T cells on sensitivity to induction of EAE relapses.

It is known that both bacterial superantigens, such as SEB, and TNF-α induce temporary increases in EAE symptoms, relapses, in mice recovering from an initial EAE episode. These relapses may resemble very much the relapsing and remitting form of multiple sclerosis in man. Such mice provide, therefore, an excellent opportunity for the study of the effect of therapeutic measures.

E. SEB-induced EAE relapses

The effect of TGF-β1/MBP T cells on SEB-induced relapses was first investigated. Since the severity of the initial EAE episode might influence the relapse rate, we compared control mice receiving no T cells with mice receiving both TGF-β1/MBP T cells and anti-TGF-β1, a mAb that at least temporarily neutralizes the protective effect of the TGF-β1/MBP T cells. Similar to the previously used specific anti-TGF-β1 (4A11) (Chen et al, 1998), the mAb that neutralizes all three TGF-βs (2G7) prevented the protective effect of the T cells seen immediately after transfer. A single injection at the time of TGF-β1/MBP T cell administration partially transiently reversed the protective effect of the T cells, but the protection by the T cells became significant again after the effect of the mAb wore off and the EAE severity in this group of mice became like that of the mice receiving the T cells alone (**Figure 5**). Two weeks after T cell transfer, the mice in this experiment received an injection of SEB ip. The incidence of relapses in control mice under such circumstances was previously shown to be ~50% (Crisi et al, 1995). In the experiment shown in **Table 2**, 3 out of 8 control mice relapsed, and 0 out of 11 in the TGF-β1/MBP T cell treated mice, indicating that recipients of TGF-β1/MBP T cells were protected from SEB-induced relapse at this time

In another experiment, the SEB injection was given much later after recovery from EAE, i.e., 6-7 weeks after T cell transfer. The results in **Figure 6A** show that the EAE severity in the control (no T cells) group had recovered to a mean of ~1.3, while barely any remaining disease was seen in the TGF-β1/MBP T cell treated recipients. Nevertheless, on injection of SEB, relapses of similar incidence (**Table 2**) and severity (**Figure 6A**) were induced in both groups.

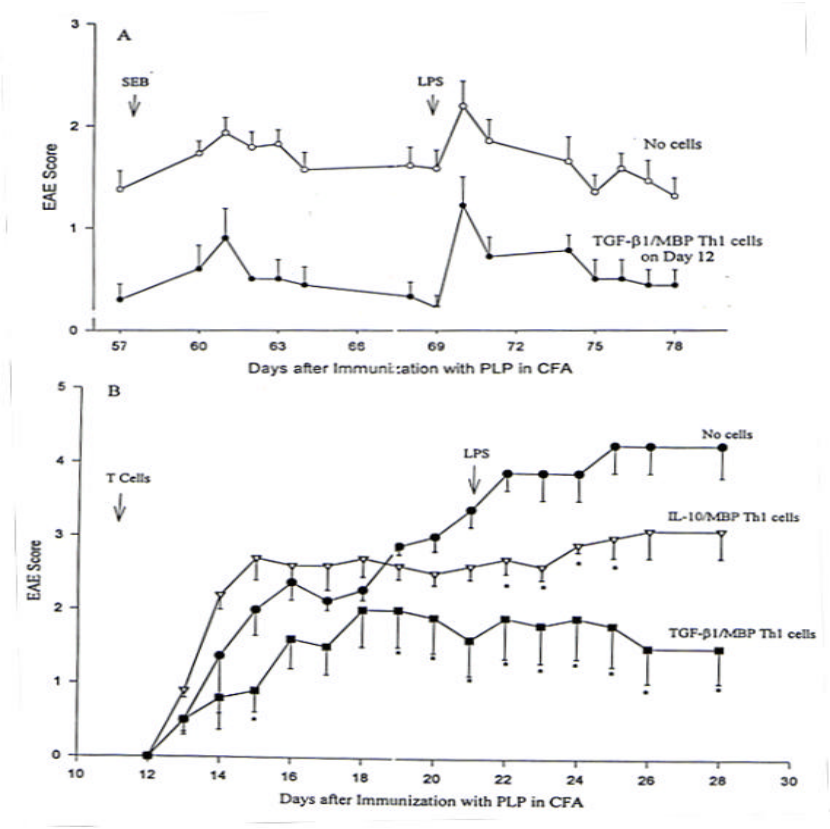

Figure 6. Comparison of the effects of IL-10 and TGF-β1/MBP T cells on SEB and LPS induced EAE relapses. A): T cells were injected 12 days after immunization with PLP and the mice treated with anti-TGF-β mAb as described for **Figure 5**. SEB (0.5 μg /mouse, ip) and LPS (1 μg/mouse, ip) were given 45 and 56 days after T cell transfer, respectively. O¾O No cell control (n=13); ●¾● TGF-β1/MBP Th1 cells (n=10). B): TGF-β1/MBP Th1 cells were injected 11 days after PLP in CFA. LPS (1 μg/mouse, ip) was injected 10 days after cell transfer. ●¾● No cell control (n=5); ¾ IL-10/MBP Th1 cells (n=5); ■¾■ TGF-β1/MBP Th1 cells (n=5).* Statistically different from control (no cells) group, $p<0.05$.

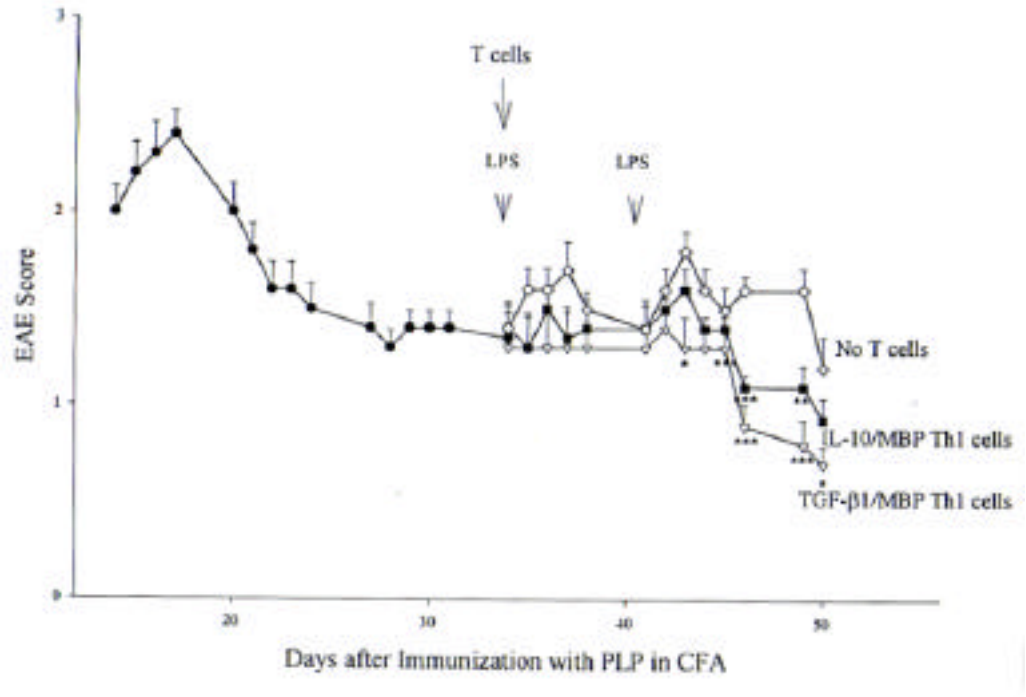

Figure 7. Effect on LPS induced EAE relapses of IL-10 or TGF-β1/MBP Th1 cells injected 5 weeks after induction of EAE. After partial recovery from EAE, mice were injected on day 34 with LPS (1 μg, ip) and with 3 x 10^6 cytokine transduced MBP Th1 cells. On day 42, all the mice were again injected with LPS (5 μg, ip). ◊¾◊ No cells (n=8); ■¾■ IL-10/MBP T cells (n=8); ¾ TGF-β1/MBP T cells (n=8); ●¾● Mice prior to injections of T cells (n=24). Statistical significance: * p<0.05; ** p<0.01; *** p<0.001, compared to mice not injected with T cells.

F. LPS induced EAE relapses

In previous studies, we have shown that TNF-α induced relapses were prevented by injection of IL-10, while SEB induced relapses were more effectively prevented by TGF-β injections (Crisi et al, 1995). Since gram negative bacterial endotoxin, LPS, induces the rapid release of TNF-α, we studied the effect of IL-10/MBP and TGF-β1/MBP T cells on the incidence and severity of EAE relapses induced by LPS. As can be seen from the results in **Figure 6B** and in **Table 2**, LPS (1 μg), injected on day 21 after immunization with PLP in CFA (or 10 days after transduced T cell transfer), caused an exacerbation of EAE severity in control (no T cells) mice. In this experiment the overall EAE severity was somewhat greater and the rate of recovery somewhat slower than in most other experiments. An additional group that received untransduced MBP specific T cells (not shown) had a slightly higher severity of EAE than the no T cell control and all of these mice died after injection of LPS. At the time of LPS injection, recipients of IL-10/MBP T cells were beginning to show a somewhat lowerEAE severity than the controls and the effect of LPS was minimal (**Figure 6B** and **Table 2**). The TGF-β1/MBP T cell recipients had significantly less disease than the other groups and failed to show a significant effect after LPS injection (**Figure 6B** and **Table 2**). An additional group of mice received both IL-10/MBP and TGF-β1/MBP T cells, but the protective effects of these combined transduced T cells were not additive (not shown). In the experiment shown in **Figure 6A**, LPS was injected 8 weeks after T cell transfer. As seen in **Table 2** and in **Figure 6A**, under these conditions LPS induced similar relapses in control and TGF-β1/MBP T cells treated mice. Thus, in these mice, in which cDNA for TGF-β1 could no longer be detected in spinal cords (**Figure 2**), there was no protection against EAE relapses.

In an additional experiment, shown in **Figure 7**, TGF-β1/MBP or IL-10/MBP T cells were injected at the same time as LPS into mice that had partially recovered from PLP induced EAE. Cells (2x10^6, iv) and LPS (1 μg, ip) were injected on day 34 after immunization with PLP in CFA. A second injection of LPS (5 μg) was given 1 week later. In the control group, each injection of LPS induced a slight increase in the EAE score, which lasted only a few days. In the mice receiving TGF-β1 transduced cells, no relapse of the EAE could be detected. In the mice receiving IL-10 transduced T cells, the EAE relapses were somewhat less marked than in the control mice. The

recovery after day 45 was accelerated in both the transduced T cell-treated as compared to the control group of mice (**Figure 7**). On day 9-16 after cell transfer, the cDNA of the transduced cytokine was detectable in the spinal cord of a large percentage of the mice receiving TGF-β1/MBP cells and again a somewhat lower percentage of the mice receiving IL-10/MBP cells (**Figure 2B**). These results show that TGF-β1/MBP T cells can enter the CNS and protect against exacerbations of EAE, even when given late during the course of the disease.

III. Discussion

The present results confirm our previous findings (Chen et al, 1998) that latent TGF-β1 transduced MBP-specific Th1 cells protect against PLP-induced EAE in (SJL x BALB/c) F1 mice, even when injected shortly after the onset of disease. When left untransduced, the same Th1 cells slightly increase the severity of actively induced EAE (Chen et al, 1998), and induce adoptive EAE in BALB/c mice (Abromson-Leeman et al, 1995). It should be noted that PLP in CFA was used for the induction of EAE, so as to avoid having MBP depots present in any other sites of the body, possibly detaining MBP-specific T cells from reaching the CNS (Chen et al, 1998).

Clearly, the only difference between the transduced and the untransduced cloned T cells is the enhanced production of TGF-β1. The transduced cells remain Th1, because they produce mRNA for TNF, LTα, LTβ and IFN-γ, and not for IL-4 or IL-10 (Chen et al, 1998). Even though this Th1 cytokine profile is unaltered after transduction with TGF-β1, the cells lose their capacity to aggravate EAE in the recipients, and instead significantly ameliorate the development of EAE. Therefore, the functional properties of these cells *in vivo* have been changed by the engineered production of latent TGF-β1. In both EAE and experimental asthma, the protective effect of TGF-β1 transduced T cells is abrogated by the simultaneous injection of neutralizing anti-TGF-β, which only interacts with active TGF-β (Chen et al, 1998; Hansen et al, 2000). It should be noted that, under normal conditions, most cells including T cells only produce latent TGF-β, i.e., TGF-β from which the latency associated protein (LAP) must be removed to uncover the receptor binding region before it exerts any biological activity (Wakefield et al, 1988). In inflammatory infiltrates this most likely occurs by enzymes such as plasmin and/or acidification in macrophages (Nunes et al, 1995; Godar et al, 1999). In addition, several other proteins have been shown to be capable of removing the LAP from TGF-β, such as thrombospondulin (Ribeiro et al, 1999) and the integrin αvβ6 (Munger et al, 1999).

TGF-β may affect autoimmune disease through down regulation of: 1) TNF-α and LT production (Espevik et al, 1987; Stevens et al, 1994); 2) responses to IL-12 (Pardoux et al, 1997); 3) macrophage and microglia activation (Nelson et al, 1991; Vodovotz et al, 1993; Lodge and Sriram, 1996); 4) cytokine enhanced class II MHC expression (Epstein et al, 1991); and 5) migration of T cells into the CNS (Santambrogio et al, 1993; Fabry et al, 1995). TGF-β induces the synthesis of IL-10 by macrophages (Maeda et al, 1995; Kitani et al, 2000), but the present results suggest that this is unlikely to be the mechanism by which TGF-β1/MBP T cells protect against EAE, since IL-10/MBP T cells are less effective. TGF-β also stimulates its own production (Fiorelli et al, 1994) and, therefore, a few TGF-β1/MBP T cells retained in an infiltrate on the basis of their specificity for myelin protein, may cause oligodendrocytes and macrophages in their vicinity to produce more TGF-β. An additional mechanism by which TGF-β may influence autoimmunity is through the promotion of immunoregulatory $CD8^+$ T cell development (Quere and Thorbecke, 1990; Rich et al, 1995; Powrie et al, 1996; Thorbecke et al, 1999).

The primary mechanism by which IL-10 protects against the development of autoimmune diseases, such as CIA, is thought to be through inhibition of the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Walmsley et al, 1996; Kim et al, 2000) and of chemokines, such as MIP-1α and MIP-2 (Kasama et al, 1995). Moreover, IL-10 directs T cells away from harmful Th1 responses and associated IgG2a antibody formation, into the direction of Th2 (Kim et al, 2000; Stevens et al, 1988). Indeed, the resistance of IL-10 transgenic mice to induction of EAE is attributed to the inhibition of Th1 responses in such mice (Cua et al, 1999). Similar to TGF-β, IL-10 counteracts the activation of macrophages and in this respect synergizes with TGF-β (Oswald et al, 1992). In contrast to TGF-β, however, IL-10 fails to inhibit NO production by macrophages induced by an extraneous source of TNF-α (Bogdan et al, 1991; Corradin et al, 1993). Both cytokines counteract the upregulation of class II MHC and of FASL expression by IFN-γ (de Waal Malefyt et al, 1991; Epstein et al, 1991; Arnold et al, 1999), and inhibit the expression of contact sensitization in sensitized mice (Epstein et al, 1991; Ferguson et al, 1994). It is, therefore, not immediately clear why TGF-β1/MBP T cells are much more effective in our model of EAE than IL-10/MBP T cells, and why the effects of these cells given simultaneously are not additive. Neither of the transduced cloned T cells has been affected in its ability to proliferate in response to MBP, and both are detectable in spinal cords after transfer, although the persistence of the IL-10/MBP cells is somewhat shorter. In the airway hyper-reactivity model, transfer of OVA-specific IL-10-transduced T cells results in pronounced inhibition of airway hyper-reactivity (Oh et al, unpublished observations). The differences in the effectiveness of OVA-transduced cells in these systems may reflect differences in the effects of IL-10 on the Th2 effector cells mediating the airway hyper-reactivity vs the Th1 cells mediating EAE, or in the effects of IL-10 on APCs in the two sites. Another possibility is that the IL-10 produced by the transduced T cells inhibits antigen presentation (van

der Veen and Stohlman, 1993; Frei et al, 1994; de Vries, 1995) to themselves *in vivo*, resulting in a reduced proliferation of these T cells which affects their performance in the more chronic situation of the EAE model, but is less important in acute airway hyper-reactivity.

It has been reported that, unlike TGF-β, IL-10 also has immune-stimulating effects on CD8 T cells (Chen and Zlotnik, 1991; Balasa et al, 1998; Groux et al, 1999) and B cells (Briere et al, 1993). Moreover, while IL-10 inhibits pro-inflammatory cytokine production in macrophages, it does not affect endothelial cells (Sironi et al, 1993) or dendritic cells from rheumatoid synovial fluid (MacDonald et al, 1999). It is possible that the greater inhibition of NO production exerted by TGF-β1 is of importance, as NO has been linked to damage of the CNS in EAE in various studies (Lin et al, 1993; Okuda et al, 1995; Waldburger et al, 1996). It should also noted that in transgenic mice, IL-10 expressed under control of an MHC class II promoter causes enhanced susceptibility to Leishmania infection, while IL-10 expressed only in T cells does not have this effect (Groux et al, 1999). In this respect it is perhaps relevant that in the present studies, the enhanced cytokine production is only in a small population of transferred T cells. While the transduced cytokines, latent TGF-B1 and IL-10, were produced by the T cells to approximately the same levels (in ng amounts), it is not sure what the effective concentrations required *in vivo* might be for each of these cytokines, or how much of the latent TGF-β1 produced by the cells becomes activated at the sites where it exerts its effect. We have not been able to obtain a higher production of IL-10 in the T cells.

In view of the consideration that clinical application of transduced T-cell therapy in humans would have to be performed after initiation of disease, the possibility of affecting relapses of EAE was also investigated in these studies. The relapses studied here were induced by injection of TNF-α and IFN-γ inducing agents, which may mimic clinical situations in which relapses of demyelinating disease are known to occur, such as during infections (Edwards et al, 1998; Metz et al, 1998). Both SEB and LPS induce a burst of TNF-α production and, although unlike IL-10, TGF-β cannot overcome the effects of injected TNF-α, TGF-β does inhibit TNF-α production (Espevik et al, 1987), which may be an important aspect of the inhibitory effect on these relapses. SEB, in addition, stimulates Vβ3 and Vβ8 T cells (Marrack and Kappler, 1990), and causes production of large amounts of T cell cytokines.

In previous studies on EAE with injected cytokines, we found that IL-10 protected against TNF-α induced relapses, while TGF-β was more effective against SEB induced relapses (Crisi et al, 1995). In the present study, TGF-β1/MBP T cells prevent both the SEB- and LPS-induced increments in EAE scores, and both IL-10/MBP and TGF-β1/MBP T cells ameliorate EAE relapses induced by injection of LPS during the interval when the transduced T cells are still detectable in the CNS. More importantly, injection of the MBP-specific T cells at the time of the induction of the EAE relapse also results in significant protection, particularly by the TGF-β1 transduced cells.

It is of interest that, even though TGF-β1 producing cells are known to be relatively abundant in mucosal linings in the lung (Magnan et al, 1997; Vignola et al, 1997), injection of TGF-β1/OVA T cells nevertheless significantly protects against the local inflammatory responses accompanying airway hyper-reactivity (Hansen et al, 2000). Apparently, a protective effect can only be obtained with these transduced T cells if they localize at the site of the inflammation. In the EAE model, this can only be obtained with T cells specific for a myelin component and activated *in vitro* prior to cell transfer. It has been shown that activated T cells which penetrate the blood-brain-barrier during EAE have upregulated adhesion molecules on their surfaces, such as VLA-4 and LFA-1, and that the presence of adhesion molecules on cloned T cells influences their capacity to transfer EAE to recipient mice (Kuchroo et al, 1993; Barten et al, 1995). In addition, contact of microvascular endothelial cells with activated T cells causes the enhancement of VCAM-1 and ICAM-1 expression on the endothelial cells (Lou et al, 1996). Thus, antigen stimulation of MBP-specific T cells is needed before they can either transfer EAE (Kuchroo et al, 1993) or protect against EAE, when transduced with TGF-β (Chen et al, 1998). It is somewhat surprising that activated T cells of unrelated specificity (KLH or OVA), with the numbers of cells used in the present study, cannot protect against EAE, even though they produce large amounts of latent TGF-β *in vitro*. In previous experiments it was shown that the TGF-β cDNA from OVA/TGF-β1 cells was barely detectable in the spinal cord 12 days after cell transfer (Chen et al, 1998). The results suggest that, in the absence of specific antigen within the CNS, T cell numbers, proliferation and/or continued localization within infiltrates during the course of the EAE must have been insufficient when compared to those of MBP-specific cells. In the asthma model, however, the accumulation in the lung of T cells of any specificity can be obtained by allowing the mice to inhale the relevant antigen (Tsuyuki et al, 1997). Therefore, while there is no protective effect of TGF-β1/KLH cells against airway hyperreactivity in OVA sensitized and challenged mice, partial protection with such cells is obtained in mice sensitized by inhalation of OVA alone and challenged with OVA + KLH.

Prolonged systemic treatment with active TGF-β is contraindicated in patients, because it induces liver fibrosis and glomerulosclerosis (Calabresi et al, 1998), as also seen in transgenic mice (Clouthier et al, 1997). A major advantage of the present approach to control autoimmune disease is that the TGF-β1 constitutively produced in the transferred cells is latent rather than active, and is therefore unlikely to have these side effects. Under normal conditions, latent TGF-β is present

ubiquitously, in platelet α granules (Fava et al, 1990), as well as attached to the matrix of connective tissue (Heine et al, 1990; Munger et al, 1997; Evanko et al, 1998) and to γ-globulin in the serum of mice (Rowley et al, 1995) and humans (GJT, CH and GMH, unpublished observations). The latent TGF-β1 produced by the antigen specific T cells in inflammatory infiltrates in the CNS is apparently activated, possibly by neighboring macrophages in the lesions (Nunes et al, 1995). Indeed, an increase in active TGF-β1 can be detected in the asthma model in bronchoalveolar lavage fluid harvested from mice receiving TGF-β1/OVA T cells one day after measurement of airway hyper-reactivity (Hansen et al, 2000). In contrast, levels of active TGF-β are low in bronchoalveolar lavage fluid from OVA-immunized mice receiving KLH/TGF-β rather than OVA/TGF-β Th1 cells. These data indicate that the TGF-β1 transduced OVA-specific T cells reach the lung in mice that have been challenged intranasally with OVA and that the latent TGF-β1 secreted by the T cells is activated in the inflammatory environment created by OVA-specific Th2 cells, either by macrophages via interaction with plasmin (Munger et al, 1997; Godar et al, 1999) and/or betaglycan (Chong et al, 1999), or by interaction with other known TGF-β activating moieties, such as thrombospondin-1 (Ribeiro et al, 1999) or αvβ6 (Munger et al, 1999), the latter of which is prominently represented in epithelial cells in the lung.

The results so far obtained with TGF-β1 transduced T cells indicate that production of TGF-β1 confers immune-modulating properties on auto-reactive T cells, that allow them to control the behavior of other inflammatory cells in their immediate vicinity. We propose that the genetic engineering of auto-reactive T cells with latent TGF-β, or up-regulating their ability to produce TGF-β by other means, such as through inhibition of CD26 (dipeptidyl peptidase IV) (Kahne et al, 1999) may represent a clinically viable approach to the treatment of autoimmune diseases.

IV. Materials and Methods

A. Mice

(SJL x BALB/c) F1 hybrid mice, 6-8 weeks old females, were purchased from the Jackson Lab. (Bar Harbor, ME).

B. Studies on EAE

SJL x BALB/c mice were injected sc. with 200 μg PLP peptide 139-151 (Molecular Dynamics, Sunnyvale, CA), emulsified in incomplete Freund's adjuvant containing 200 μg killed H37RA *Mycobacteria tuberculosa*. The mice received 200 ng pertussigen iv, 24 and 48 h later. The EAE was scored (double blind read) as follows: 1 = limp tail; 2 = partial hind leg paralysis; 3 = total hind leg paralysis; 4 = hind and front limb paralysis; 5 = moribund (Santambrogio et al, 1993). Predictable EAE relapses (Crisi et al, 1995) were induced by injection of *Staphylococcus* enterotoxin B (SEB, Toxin Technology, Sarasota, FL), 0.5 μg ip, or of LPS (lipopolysaccharide B, *E*. coli 0111:B4 **,** Difco Labs, Detroit, MI), 1-5 μg ip. Transduced and control cloned T cells (2-3 days after activation with the relevant antigen *in vitro*, 3 x 10^6 cells/mouse) were injected iv into mice which had been immunized with PLP 12 days earlier. Differences between groups of mice for mean EAE severity were evaluated by Student's t test; for EAE incidence by Chi^2 test.

C. T cell clones

The MBP-specific cloned T cells were derived from BALB/c mice immunized with MBP in CFA (Abromson-Leeman et al, 1995) and were donated by Dr. M. Dorf (Dept. of Pathology, Harvard U. Med. School). Cells were activated by exposure to MBP peptide 59-76 (10μg/10^6 cells, Peptide Synthesis, Keck Biotechnology Resource Center, New Haven, CT) in the presence of antigen presenting cells (APC, 5 x 10^6 γ-irradiated spleen cells). Keyhole limpet hemocyanin (KLH) specific (D3) and ovalbumin (OVA) specific (BOT.A3) BALB/c Th1 cell clones were grown and activated as described previously (Rizzo et al, 1992). For experiments in which TGF-β contents of supernatants were to be measured, cells received 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN) instead of serum in the medium. All the T cell clones were stimulated every 2-3 weeks by the corresponding antigens: MBP (10 μg/ml), KLH (1 μg/ml), OVA (10 μg/ml).

D. Studies on airway hyper-reactivity:

Immunization Protocol of Normal BALB/c Mice to Induce Airway Hyper-reactivity. TGF-β producing cells Th1 cells were also transferred into OVA-immunized BALB/c mice. BALB/c mice were immunized with OVA i.p. (50 μg) complexed with aluminum potassium sulfate (alum) on day 0, and intranasally with 50 μg OVA in 50 μl of PBS or 50 μg OVA and 25 μg KLH in 50 μl of PBS on days 7, 8 and 9. Some mice received TGF-β1 producing OVA or KLH specific Th1 cells iv (2.5 x 10^6 cells/mouse) on day 7. Airway hyper-reactivity to inhaled methacholine was measured 24 h after the last intranasal dose of OVA (day 10). Lung fixation was performed the following day.

E. Measurement of airway responsiveness

Airway responsiveness was assessed as described previously (Hansen et al, 2000) by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211, Buxco Electronics Inc., Troy, NY). Pulmonary airflow obstruction was measured by Penh using the following formula: Penh = (Te/RT-1) x (PEF/PIF), where Penh=enhanced pause (dimensionless), Te=expiratory time, RT=relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s). Enhanced pause (Penh), minute volume, tidal volume, and breathing frequency were obtained from chamber pressure, measured with a transducer (model TRD5100) connected to preamplifier modules (model MAX2270) and analyzed by system XA software (model SFT 1810). Measurements of methacholine responsiveness were obtained by exposing mice for 2 min to NaCl 0.9% (Portable Ultrasonic, 5500D, DeVilbiss Health Care, Inc. Sommerset, Pennsylvania), followed by incremental doses (2.5-40 mg/ml) of aerosolized methacholine and monitoring Penh. Results were expressed for each methacholine concentration as the percentage above baseline Penh values after NaCl 0.9 % exposure.

F. Transduction of T cell clones

The cDNA (base pairs 352-1550) encoding murine TGF-β1 (generously provided by DNAX, Palo Alto, CA) was

subcloned into the pMFG retroviral vector as previously described (Dranoff et al, 1993). The cDNA of murine IL-10 (base pairs 77-623) was similarly subcloned into the pMFG vector. CRIP-TGF-β and CRIP-IL-10 packaging cells, producing the replication defective retrovirus, were generated as reported previously (Danos and Mulligan, 1988). The titers of the retroviruses were 0.5 copies as determined by Southern analysis. No replication competent virus was detected using the his mobilization assay (Hartman and Mulligan, 1988). Transduction of T cell clones was done by co-culture with packaging cells for 48 h in the presence of 2 μg polybrene per ml (Cepko and Pear, 1997). The packaging cells were γ-irradiated (2800 r) and plated in a 24-well plate (2 x 10^5 cells/well). Four h later, when the fibroblasts had completely adhered to the well, recently activated cloned T cells (10^6/well) were added. The T cells were then cloned by limiting dilution in 96-well plates, using γ-irradiated BALB/c spleen cells (5 x 10^5/ml) as feeder cells. Clones were expanded until sufficient amounts of DNA could be obtained for PCR analysis.

H. PCR for detection of cytokine cDNA

DNA extraction was done according to the instructions in the Promega Wizard Genomic DNA purification kit. Primers used for the detection of TGF-β1 cDNA (Clontech, Palo Alto, CA) were: FP, (5') GCCCTGGACACCAACTATTGCT and RP, (3') AGGCTCCAAATGTAGGGGCAGG. They correspond closely (with one base pair difference) to the mouse TGF-β1 sequences, 1187-1208 and 1347-1326, respectively. The PCR program followed was: 95°C 5 min; 94°C 30', 55°C 30',72°C 1 min, 40 cycles; 72°C 10 min, using 1 μg sample DNA per reaction. Approximately 3% of the cloned T cells proved positive for the cDNA of TGF-β1. Primers used for the detection of IL-10 cDNA were FP, (5') TCCTTAATGCAG GACTTTAAGGGTTACTTG and RP, (3') GACACCTTGGTCTTGG AGCTTATTAAAATC, which correspond to the cDNA sequences 270-309 and 527-508, respectively. The same PCR program was used as for amplification of TGF-β1 cDNA. For both TGF-β1 and IL-10, the primers were chosen to span an intron, such that endogenous DNA would not be amplified. Positive and negative controls were always included(Chen et al, 1998).

To control for PCR conditions and DNA quality, PCR for MMTV-LTR was performed on spinal cord samples using the forward primer: (5') CTACACTTAG GAGAGAAGCAGCCA and the reverse primer: (3') CTTACTTAAACCTTGGGAACCG CAAG (Zhang et al, 1996).

I. Cytokine production

RNA was extracted from 2 x 10^6 cloned T cells, 2-3 days after stimulation with antigen, using the RNA STAT-60 isolation kit (Tel-Test, Inc., Friendswood, TX). Cytokine mRNAs produced by antigen activated cloned T cells were quantified by multiprobe ribonuclease protection assay, using two sets of cytokine probes according to the manufacturer's instructions (PharMingen, San Diego, CA).

The biological activity of TGF-β was assayed in supernatants from 10^6 cells per ml, cultured for 24 h in serum-free medium, by its activating effect on the plasminogen activator inhibitor-1 (PAI-1) promotor linked to the firefly luciferase reporter gene, transfected into mink lung epithelial cells(Abe et al, 1994). The assay cells were a generous donation from Dr. D. B. Rifkin (Dept. Cell Biology, NYU School of Medicine). Samples were assayed with and without activation of latent TGF-β by treatment with acid (0.1M HCL at 4°C for 60 min).

The protein content of TGF-β1 was assayed by ELISA in Immulon 4 flat bottom plates, coated with 5 μg/well of a monoclonal anti-TGF-β{12H5 (Lucas et al, 1990)}, donated by Dr. B. M. Fendly, Genentech Inc.), using natural TGF-β1 (Genzyme, Cambridge, MA) as a standard, and biotinylated mAb. to TGF-β1 (R&D systems, Minneapolis, MN), streptavidin-peroxidase (Zymed, South San Francisco, CA) and OPD substrate (Sigma, St. Louis, MO) as developing reagents. IL-10 was assayed by ELISA with the use of the Endogen kit (Woburn, MA).

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