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IL-4 and IL-10 Are Both Required for the Induction of Oral Tolerance[1]

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Protection from the development of experimental autoimmune uveitis (EAU) can be induced by feeding mice interphotoreceptor retinoid binding protein before uveitogenic challenge with the same protein. Two different regimens are equally effective in inducing protective tolerance, although they seem to do so through different mechanisms: one involving regulatory cytokines (IL-4, IL-10, and TGF-β), and the other with minimal involvement of cytokines. Here we studied the importance of IL-4 and IL-10 for the development of oral tolerance using mice genetically engineered to lack either one or both of these cytokines. In these animals we were able to protect against EAU only through the regimen inducing cytokine-independent tolerance. When these animals were fed a regimen that in the wild-type animal is thought to predominantly induce regulatory cells and is associated with cytokine secretion, they were not protected from EAU. Interestingly, both regimens were associated with reduced IL-2 production and proliferation in response to interphotoreceptor retinoid binding protein. These findings indicate that both IL-4 and IL-10 are required for induction of protective oral tolerance dependent on regulatory cytokines, and that one cytokine cannot substitute for the other in this process. These data also underscore the fact that oral tolerance, manifested as suppression of proliferation and IL-2 production, is not synonymous with protection from disease. *The Journal of Immunology,* **1999, 162: 2613–2622.**

Mucosal surfaces are one of the most important entry routes for pathogens into the body, and the immune system had to evolve in a way to protect these surfaces, to generate quick and effective responses to avoid the penetration and seeding of pathogens. Also, our nutrition and reproduction depend on the mucosal immune system being tolerant to certain types of stimuli, such as food Ags and HLA Ags, respectively. Mucosal tolerance is the phenomenon by which contact with Ag through the mucosal tissues results in decreased responses to the same Ag when presented later by parenteral immunization. The ability of the gut-associated lymphoid tissue to effect this regulatory function has been recognized for many years (1). It is noteworthy that this property is not restricted to the gut mucosa but is inherent to any mucosal surface in the body, such as that surrounding the respiratory tract and the genito-urinary tract (2–6). The ability of orally induced tolerance to prevent the development of autoimmune diseases has been explored in such autoimmune disease models as collagen arthritis, experimental allergic encephalomyelitis (EAE),[4] experimental autoimmune uveitis (EAU), diabetes, and myasthenia (7–11).

The use of oral tolerization in the clinic would only be possible under either one of two conditions: the eliciting Ag is known (in this case treatment may need to be customized for each patient), or feeding of an Ag present in the target organ and likely to be exposed during the inflammatory process can generate suppressor cells secreting Ag-nonspecific anti-inflammatory cytokines that, acting locally, would decrease inflammation. This phenomenon (bystander suppression) is of utmost importance from the clinical point of view, since in most cases the triggering Ag in human autoimmune diseases is not known. Investigators have reported that Lewis rats fed OVA and immunized with MBP and OVA given separately s.c. were protected from EAE (12). The protective effect could be adoptively transferred by $CD8^+$ cells from OVA-fed animals into MBP- plus OVA-injected rats (12). Furthermore, it was shown that oral tolerization with mouse MBP suppressed EAE in the SJL mouse, in which autoimmunity to proteolipid protein appears to play a primary role, suggesting that Ag-driven bystander suppression following oral tolerization with autoantigens may play some role in suppression of EAE following oral tolerization with MBP in this model (13).

Oral tolerance has been used successfully as an immunotherapeutic approach to many experimental models of autoimmune diseases. Although clinical trials of oral tolerance in multiple sclerosis and arthritis did not show positive results, a clinical trial using S-Ag as the oral tolerogen in the treatment of autoimmune uveitis rendered very encouraging results (14). To improve on the efficacy

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[4] Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; EAU, experimental autoimmune uveitis; MBP, myelin basic protein; IRBP, interphotoreceptor retinoid-binding protein; 3× + IL-2, three feedings of 0.2 mg of IRBP followed by one injection of IL-2 at priming; PP, Peyer's patch; 5×, three feedings of 0.2 mg of IRBP ; KO, knockout; PTX, pertussis toxin; wt, wild-type; LN, lymph node.

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of this alternative approach to the treatment of autoimmunity it becomes imperative to understand the mechanisms involved in the development and maintenance of tolerance by oral administration of Ag. It has been suggested that anergy or deletion of Ag-specific cells and active suppression are responsible for the development of oral tolerance. It is important for us to determine parameters are involved in defining the dominant mechanism and to establish the participation of cytokines in the process. It is therefore important to understand the basic mechanisms involved in this phenomenon by taking advantage of the animal models available. It has been suggested that feeding of small amounts of protein would result in tolerance by active suppression, whereas a high dose would result in anergy or T cell deletion in a similar fashion to tolerance induced by i.v. injection of Ag (15, 16). It is likely that these mechanisms are overlapping and will occur concomitantly, with one or the other predominating. It was also proposed that inhibition of systemic delayed-type hypersensitivity after feeding proteins is due to active suppression, whereas inhibition of systemic humoral immunity may result from T cell anergy (17–19). Using the murine model of EAU induced by immunization with interphotoreceptor retinoid binding protein (IRBP), we have shown that oral tolerance elicited by three feedings of 0.2 mg of IRBP followed by one injection of IL-2 at priming (3× + IL-2) correlates with the production of TGF-β, IL-4, and IL-10 by Peyer's patch (PP) cells. Suppression could also be elicited by 5× feeding, with minimal production of cytokines (20). This has led us to propose that tolerance induced by the 3× + IL-2 regimen involves mainly regulatory cells, whereas protection induced by the 5× regimen involves mainly anergy or deletion of the uveitogenic effector T cells (20).

To examine the importance of IL-4 and IL-10 in oral tolerance induction, knockout mice (KO) deficient for IL-4 and/or IL-10 and their normal littermates were fed IRBP using either the 3× + IL-2 or the 5× regimen and were challenged with a uveitogenic dose of IRBP. We report here that IL-4 and IL-10 KO mice were not protected against EAU by the 3× + IL-2 feeding regimen that protects their normal littermates against the development of disease. However, these same IL-deficient mice were protected by the 5× regimen.

Materials and Methods

Animals

C57BL/6 mice of either sex were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-4-deficient mice (IL-4 KO) and IL-10-deficient mice (IL-10 KO) were gifts from Drs. Klaus Rajewsky, Werner Müller, and Ralf Kühn (Institute of Genetics, University of Kohn, Kohn, Germany) and were bred to the C57BL/6 background by one of us (R.A.M.). Mice deficient in both IL-4 and IL-10 (DKO) were bred by R.A.M. from the single KO mice. Animals were kept in microisolator cages under specific pathogen-free conditions and were handled in compliance with National Eye Institute, National Institutes of Health, and Instituto de Ciêancias Biomédicas-University of Sao Paulo guidelines for animal use.

Reagents

IRBP was isolated from bovine retinas as described previously using Con A-Sepharose affinity chromatography and fast performance liquid chromatography (21, 22). IRBP preparations were aliquoted and stored at −70°C. BSA, α-methyl-mannoside, Con A, pertussis toxin (PTX), CFA, and conalbumin were purchased from Sigma (St. Louis, MO), horseradish peroxidase-streptavidin was obtained from Southern Biotechnologies Associates (Birmingham, AL). PHA-p and *Mycobacterium tuberculosis* strain H37RA were purchased from Difco (Detroit, MI). Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia (Piscataway, NJ).

Antibodies

The anti-IL-10 mAb-producing hybridomas (SX1, SX2, and 2S5) were provided by Drs. Kevin Moore and Robert Coffman (DNAX Research Institute, Palo Alto, CA). The J4-1 (rat anti-4-hydroxy-3-nitro-phenyl acetyl) was used as a control as described previously (23). Polyclonal rat IgG1 isolated from naive male rats by affinity chromatography was also used as a control in some experiments. Ab against TGF-β was obtained by immunizing chickens with recombinant TGF-β in alum. The Abs were recovered from sera by immunoaffinity chromatography using an anti-chicken Ig Ab coupled to Sepharose. TGF-β-neutralizing activity of the Ab obtained from the column was tested in a CCL-64 assay (20) using rTGF-β in different concentrations. One microgram of Ab was shown to neutralize 100 pg of TGF-β in vitro.

Cell culture medium

T cells were cultured in DMEM supplemented with 10% FCS (HyClone, Logan, UT) or 0.5% normal mouse serum obtained from syngeneic naive mice (as indicated), 10^{-5} M 2-ME, (Sigma), 2 mM L-glutamine, 0.1 mM nonessential amino acids, and vitamins (Life Technologies, Grand Island, NY) (23).

Recombinant cytokines

Recombinant human IL-2 was purchased from Boehringer Mannheim (Mannheim, Germany), recombinant murine IL-5, and IFN-γ were obtained from Genzyme (Boston, MA) or from R&D Systems (Minneapolis, MN). Recombinant murine IL-4 and IL-10 were gifts from Dr. Satwat Narula, Schering Plough (Nutley, NJ). Recombinant TGF-β was a gift from Dr. Chi-Chao Chan (National Eye Institute, National Institutes of Health) or was purchased from R&D Systems, Promega (Madison, WI), or Life Technologies.

Immunization

Mice were immunized s.c. with 100 μg of IRBP in 0.2 ml of emulsion (1/1, v/v) with CFA that had been supplemented with *Mycobacterium tuberculosis* to a final concentration of 1 mg/ml and were given 2 μg of PTX in 0.1 ml of RPMI 1640 i.p. as an additional adjuvant.

Anti-cytokine treatment of mice

Mice fed IRBP were given i.p. eight equal doses of the anti-IL-10 Ab mixture, J4-1 mAb (rat IgG1 anti-4-hydroxy-3-nitrophenylacetyl), or polyclonal rat IgG starting 1 day before initiation of the feeding and spread twice a week until the termination of the experiment. Mice fed or not fed IRBP (3× + IL-2 regimen) were given i.p. two equal doses of 8 mg of chicken anti-TGF-β Ab or chicken control Ab 1 day before the first feeding and 7 days after immunization with a uveitogenic dose of IRBP. Mice were given three doses of 0.5 μg of the anti-IL-4 Ab 11B.11 starting 1 day before the feeding of IRBP began and then every 3 days. Alternatively, mice were given three doses of 0.5 μg of 11B.11 every 3 days starting on day 7 after immunization with a uveitogenic dose of IRBP.

Lymphokine assays

IL-2 was measured in supernatants collected 24 h after antigenic stimulation by the HT-2 bioassay as previously described (23). Briefly, cell culture supernatants were obtained from the cell lines and clones at different time points after Ag stimulation. The supernatant dilutions were distributed in 96-well plates. HT-2 cells were added to the wells at 10^4 cells/well. Cultures were pulsed 16–18 h later with 1 μCi/well of [^{3}H]thymidine for 6 h. To confirm the presence of IL-2, anti-IL-2 Ab (S4B6, American Type Culture Collection, Manassas, VA) and/or anti-IL-4 Ab 11B11 (provided by Dr. William Paul, National Institutes of Health, Bethesda, MD) were added to some wells. The IL-2 concentration was extrapolated from a standard curve obtained by culturing HT-2 cells in rIL-2. IL-2 was also measured by ELISA using the Ab pairs from PharMingen (La Jolla, CA) and using recombinant murine IL-2 (PharMingen) as a standard. Briefly, 96-well microtiter plates (Costar, Cambridge, MA) were coated with the appropriate anti-cytokine Ab (1 μg/ml in 0.5 M sodium carbonate/bicarbonate, pH 9.6, buffer). After blocking the plates with BSA and overnight incubation with supernatants, the plates were developed using biotin-conjugated anti-cytokine Abs, followed by a 45-min incubation at 37°C. After additional washes, horseradish peroxidase-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL) was added before development with *o*-phenylene diamine. IL-4 was measured by ELISA using the Ab pairs from PharMingen. Supernatants for IL-4 measurement were collected 48 h after antigenic stimulation. IFN-γ was measured by ELISA using Ab pairs from PharMingen in supernatants collected after 48 h of antigenic stimulation. IL-10 was measured using an ELISA kit from Endogen (Boston, MA) on supernatants collected 48 h after antigenic stimulation. TGF-β was measured using a minikit from Promega on supernatants collected after 48 h of antigenic stimulation.

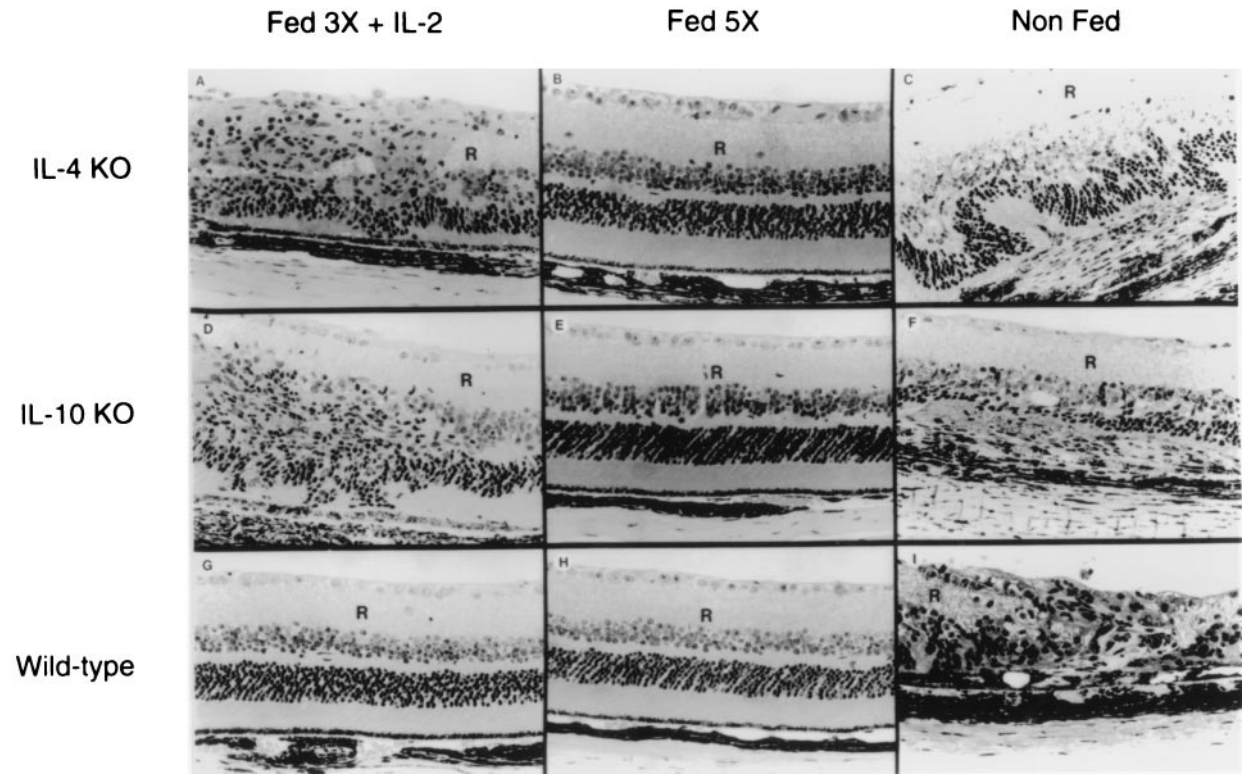

FIGURE 1. Histopathology of the eyes in IL-4 KO (*A–C*), IL-10 KO (*D–F*), and their wild-type littermates (*G–I*). All three groups of animals were protected by the 5× feeding regimen (*B*, *E*, and *H*). Unfed animals in the three groups (*C*, *F*, and *I*) presented similar degrees of disease, with comparable histopathological characteristics. A granuloma was observed in the retina (R) of an IL-4 KO mouse (*A*) and an IL-10 KO mouse (*D*) fed the 3× + IL-2 regimen. In contrast, no disease was detected in a wt mouse given the 3× + IL-2 regimen (*E*). Magnification, ×400.

Magnetic cell sorting

CD4 and CD8 cells were isolated by magnetic cells sorting using a magnetic cell-sorting magnet and microbeads coated with anti-mouse CD4 or anti-mouse CD8 Abs according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The isolation was performed using positive and negative selection columns. Briefly, PP were obtained and teased to a single cell suspension as described above. Magnetic labeling was performed with the appropriate Ab-coated microbeads for negative selection in the refrigerator for 20 min. After negatively selecting for either CD4 or CD8, the resulting population was put through a positive selection column with the respective Ab-coated microbeads. The effectiveness of the cell selection process was evaluated by FACS. The $CD4^+$ cell separation contained $<5\%$ $CD4^-$ cells and $<0.2\%$ $CD8^+$ cells. The $CD8^+$ cell separation had $<7.5\%$ $CD8^-$ cells and $<0.2\%$ $CD4^+$ cells.

Histopathology and EAU grading

Eyes were obtained 17–22 days after immunization. Freshly enucleated eyes were fixed for 1 h in 4% phosphate-buffered glutaraldehyde and transferred into 10% phosphate-buffered formaldehyde until processing. Fixed and dehydrated tissue was embedded in methacrylate, and 4- to 6-μm sections, cut through the pupillary-optic nerve plane, were stained with standard hematoxylin and eosin. Six sections cut at different levels were examined for each eye in a masked fashion by one of us (C.C.C. or L.V.R.), and the presence and extent of lesions were determined. The incidence and severity of EAU were scored on a scale of 0–4 in half-point increments according to a semiquantitative system described previously (24). Briefly, the minimal criterion to score an animal as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroid, or retina. Progressively higher grades were assigned for the presence of discrete lesions in the tissue, such as vasculitis, granuloma formation, retinal folding and/or detachment, photoreceptor damage, etc. The grading system takes into account lesion type, size, and number.

Statistical analysis of the data was performed using the Snedcor and Cochran linear trend in proportions where appropriate; $p \leq 0.05$ was considered significant.

Results

Oral tolerance against the development of EAU can be induced by an IL-4/IL-10-dependent mechanism or by an IL-4/IL-10-independent mechanism

We have previously shown that protection against the development of EAU induced by the 3× + IL-2 feeding regimen is likely to involve anti-inflammatory cytokines (IL-4, IL-10, and TGF-β), whereas protection induced by the 5× regimen might involve anergy or deletion of the uveitogenic T cells (20). To investigate the importance of IL-4 and IL-10 on the development of EAU as well as on the ability to induce oral tolerance, we fed IRBP to IL-10- or IL-4-deficient (KO) mice (either the 3× + IL-2 regimen or the 5× regimen). Unfed KO mice developed EAU that did not differ significantly in incidence from that of normal littermates and had similar histological features, indicating that IL-4 and/or IL-10 are not necessary for the induction of EAU (Fig. 1). However, disease severity was slightly lower in the KO mice than in their normal littermates. Their ability to develop protective oral tolerance also differed from that of their normal siblings. Both the 3× + IL-2 and the 5× regimens were protective in wild-type (wt) mice (C57BL/6). In contrast, IL-4 and IL-10 KO mice were protected only by the 5× regimen, but not by the 3× + IL-2 regimen (Figs. 1 and 2). Mice deficient in both cytokines (double KO) were also insensitive to the 3× + IL-2 regimen, but were protected by the 5× regimen (Fig. 3). These results reinforce the hypothesis that at least two pathways are involved in tolerance induced by mucosal presentation of Ag.

IL-10 KO mice spontaneously develop severe enterocolitis as they age. It was therefore important to address the possibility that

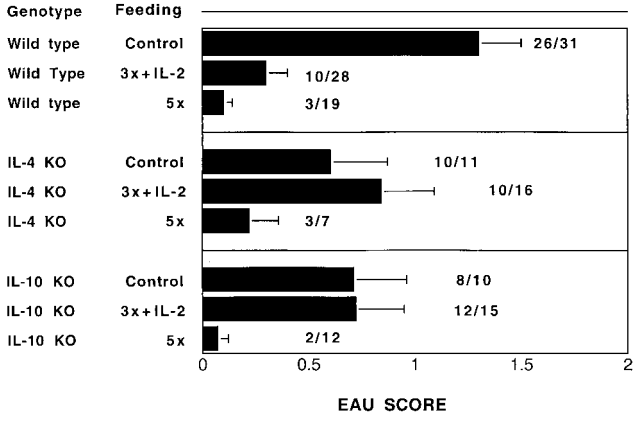

FIGURE 2. The 3× + IL-2 tolerization regimen is unable to protect IL-4- or IL-10-deficient mice against the development of EAU. IL-4 or IL-10 KO mice bred onto the C57BL/6 background are susceptible to EAU after immunization with IRBP in CFA plus an i.p. dose of PTX. Results are presented as EAU scores. The severity of EAU was scored on a scale of 0–4 in half-point increments, according to a semiquantitative system described previously (24). Briefly, the minimal criterion to score an animal as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroid, or retina. Progressively higher grades were assigned for the presence of discrete lesions in the tissue, such as vasculitis, granuloma formation, retinal folding and/or detachment, photoreceptor damage, etc. The grading system takes into account lesion type, size, and number. Incidence of disease is presented to the *right* of each bar as a function of positive animals by the total number of animals in the group. The apparent differences in disease scores between unfed KO mice and their unfed littermates are not statistically significant.

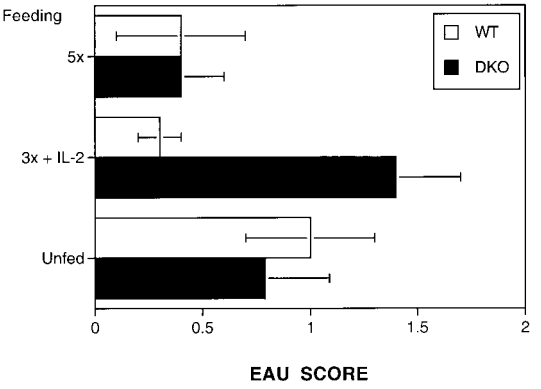

FIGURE 3. The 3× + IL-2 tolerization regimen is unable to protect IL-4- and IL-10-deficient mice against the development of EAU. Double KO mice bred onto the C57BL/6 background are susceptible to EAU after immunization with IRBP in CFA plus an i.p. dose of PTX. Results are presented as EAU scores. Disease was graded as described in Fig. 2.

their failure to develop oral tolerance with the 3× + IL-2 regimen was due to inflammatory bowel disease rather than to lack of IL-10. We therefore attempted to simulate the IL-10-deficient state by treating wt animals, fed the 3× + IL-2 regimen, with neutralizing anti-IL-10 Abs (2S5, SX1, and SX2) (25, 26). Such neutralization of endogenous IL-10 resulted in the loss of protection from EAU (Fig. 4). This result supports the interpretation that the absence of protection in IL-10 KO mice was due to their lack of IL-10 and was not a secondary effect of enterocolitis.

Because IL-4 also seems to play a role in the development of oral tolerance by the 3× + IL-2 regimen, IRBP-fed C57BL/6 mice were treated in either the afferent or efferent phase of disease induction with the anti-murine IL-4 Ab 11B.11. At the doses used, anti-IL-4 treatment does not change the effect of the 3× + IL-2 feeding regimen (Fig. 5), mostly because the anti-IL-4 treatment decreased disease severity by itself. Inhibition of disease development by 11B.11 may be more pronounced at higher doses (B. Sun, S. H. Sun, P. B. Silver, L.V.R., and R.R.C., manuscript in preparation).

The finding that both IL-4 and IL-10 seemed to be required for induction of protective tolerance was unexpected. We suggest that IL-4 is necessary as the growth factor for the regulatory cells induced by oral tolerization (27). IL-10, on the other hand, is required to promote down-regulation of the inflammatory process. To test this hypothesis, we reconstituted IL-4 and IL-10 KO mice with recombinant IL-4 or IL-10, respectively, and asked whether they would now be able to develop protection through feeding the 3× + IL-2 regimen. Various reconstitution regimens were employed covering four different doses and three different schemes of administration. In brief, we were able to reconstitute the ability of IL-10 KO mice to develop 3× + IL-2-induced protection by IL-10 replacement after cessation of feeding (300 ng/day injected i.p. in three divided doses), but we were unable to reconstitute a normal tolerogenic response in IL-4 KO mice that received IL-4 at any time or dose relative to feeding. This inability to effectively provide IL-4 replacement could be due to different reasons, among them the availability of the cytokine at the site where tolerance is being induced, probably the PP. We are currently testing the oral delivery of this cytokine. Other possibilities include a short half-life, timing of administration, and even one as prosaic as the presence of target cells, since it is possible that a lifetime absence of IL-4 may affect the development of specific cell populations.

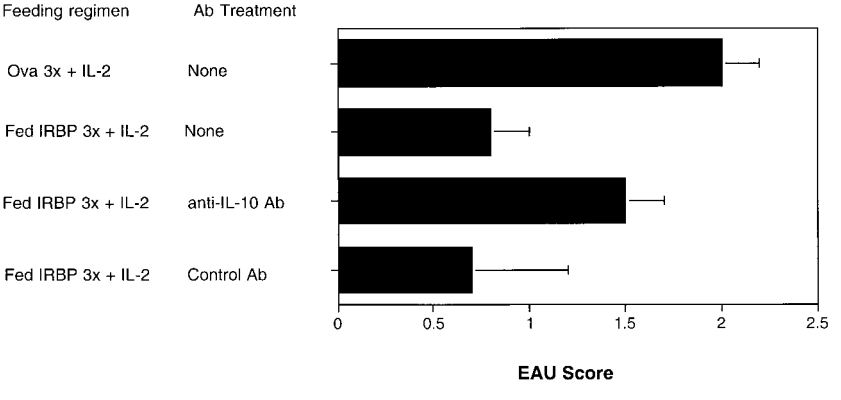

FIGURE 4. Anti-IL-10 treatment abolishes protection induced by Ag feeding. The wt B10.A mice were fed either IRBP or OVA (3× + IL-2) as described in the text. Seven animals were used in each experimental group. Some animals received anti-IL-10 Ab or an isotype control. Eight equal doses of 3 mg of Ab were given i.p. starting 1 day before the initiation of feeding (day −8) and then twice weekly throughout the course of the experiment (days −4, 0, 4, 8, 12, 16, and 18). All animals were immunized with a uveitogenic dose of IRBP (day 0).

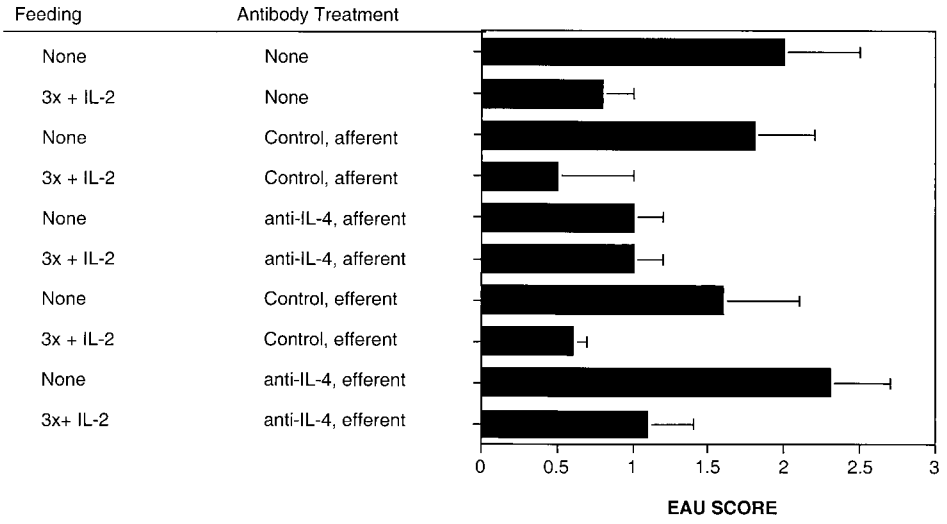

FIGURE 5. Anti-IL-4 treatment decreases EAU severity in fed and unfed mice. The wt B10.A mice were fed either IRBP or PBS (3× + IL-2) as described in the text. Five animals were used in each experimental group. Some animals received anti-IL-4 Ab (11B.11) or an isotype control. Treatment was performed in the afferent and efferent phases of the immune response as described in *Materials and Methods*. All animals were immunized with a uveitogenic dose of IRBP (day 0).

Neither IL-4 nor IL-10 is required for the decrease in lymphocyte proliferative responses induced by oral administration of Ag

It was recently reported by others that neither IL-4 nor IL-10 is essential to the development of oral tolerance under conditions known to the authors to induce active suppression (28, 29). These studies were conducted using nominal Ag as the tolerogen, and lymphocyte proliferation or Ab production as a readout. The immunological peculiarities inherent to autopathogenic Ags such as IRBP (here of bovine origin) make the evaluation of tolerance more complex, since it is assumed that some form of tolerance to autologous IRBP already exists, and that the induction of autoimmunity occurs by breaking the tolerant state. Our data show that one cannot protect IL-4 and IL-10 KO mice from EAU using the 3× + IL-2 regimen. However, when we looked at the lymphoproliferative response against IRBP in the draining lymph node (LN), we were surprised to observe that proliferation to IRBP was indeed diminished in the fed animals even though the disease scores were similar in the fed and nonfed groups (Fig. 6). A functional dissociation between in vitro proliferative responses and disease in the EAU model has been reported previously (30, 31). Several nonmutually exclusive mechanisms can be invoked to explain this dissociation: 1) lymphocyte proliferation is not a sensitive assay to detect pathogenic cells; 2) different antigenic epitopes or cell populations are involved in these two responses; while pathology by definition reflects a response to autologous epitopes, proliferation and IL-2 production are likely to reflect largely a response to nonself (bovine) epitopes of IRBP; and 3) timing differences exist, i.e., proliferation is assayed at the time that pathology is already fully developed, and most of the cells involved in the pathogenic response have migrated out of the draining LN. Regardless of which explanation is correct, it is apparent from the proliferation data that some form of tolerance was induced in the KO mice by the 3× + IL-2 regimen, which did not translate into protection from disease. It is also interesting that LN cell proliferation in response to IRBP was reduced in the unfed IL-10 KO compared with wt mice, suggesting that although the development of EAU is not altered by the absence of IL-10, this cytokine is required for the full expression of a normal proliferative response.

Cytokine profiles change after oral tolerization in animals deficient in either IL-4 or IL-10

The balance between different types of cytokines plays an important role in the development of EAU and oral tolerance (32, 33). Therefore, we decided to measure cytokines expressed in the draining LN, which is the site of immunization; in PP, which are the putative sites for the development of tolerance; and in spleen, which is required for the development of oral tolerance (34) (Fig. 7). We have postulated that protection against EAU induced by the 3× + IL-2 regimen is cytokine dependent, whereas the 5× regimen induces cytokine-independent protection. When we evaluated the cytokine production in response to IRBP in the draining LN, spleen, and PP of wt and IL-4 or IL-10 KO mice, we observed significant differences other than, of course, the lack of the respective cytokines in the KO mice.

IL-4 production, as expected, was not detected in any organ of the IL-4-deficient mice (Fig. 6*A*). We also did not observe any changes in IL-4 production in the LN or spleen of IL-10 KO mice,

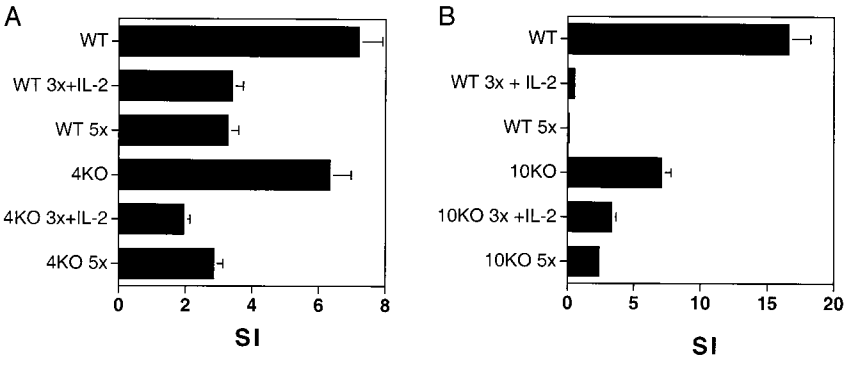

FIGURE 6. LN cell proliferation in orally tolerized IL-4 KO (*A*) and IL-10 KO (*B*) mice. Although the 3× + IL-2 feeding regimen was unable to protect against development of disease, it resulted in decreased LN proliferation in response to IRBP in both KO mice.

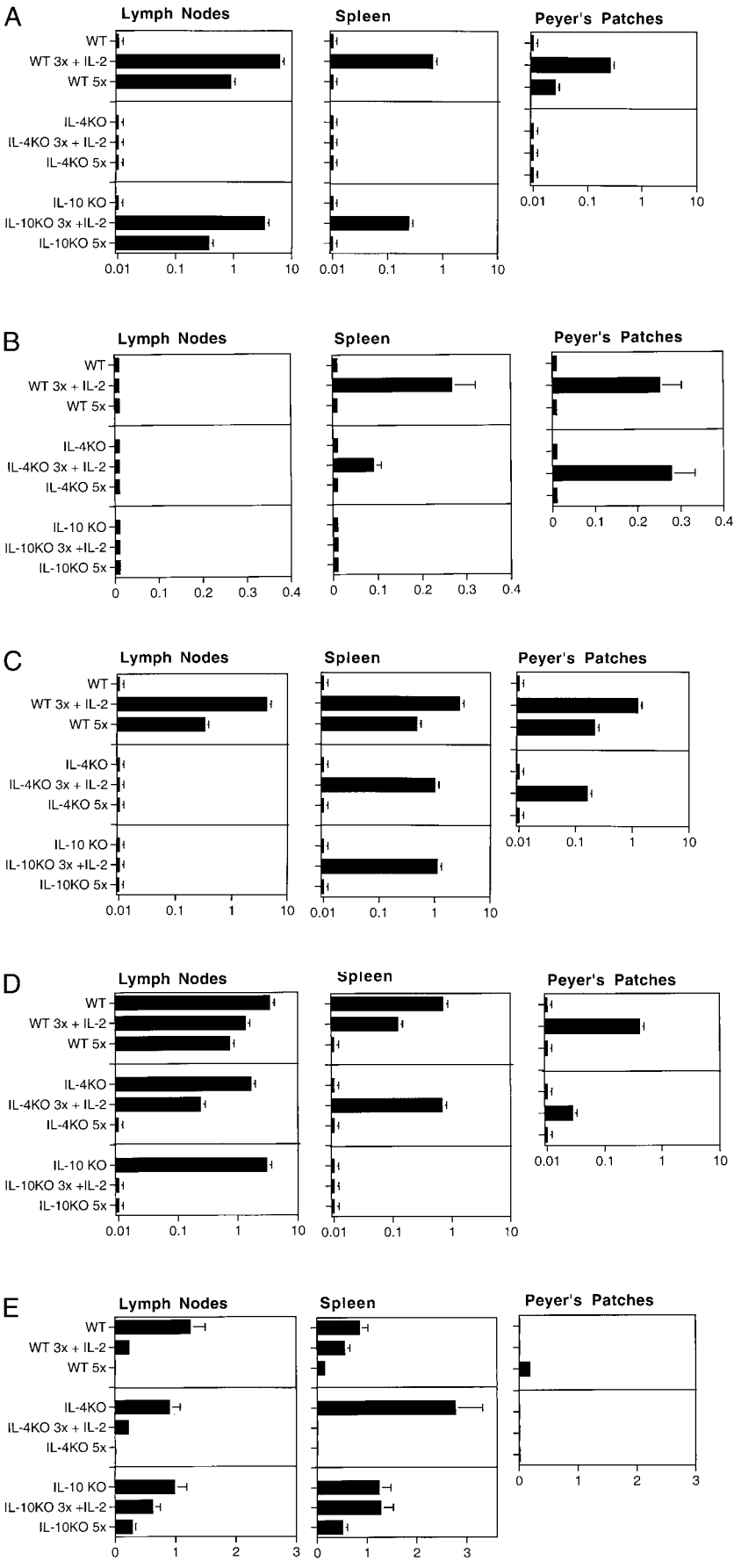

FIGURE 7. Ag-specific cytokine responses in peripheral lymphoid organs of orally tolerized IL-4 KO and IL-10 KO mice. Cytokine concentrations were measured in the culture supernatants of cells from the LN, spleen, or PP. *A*, IL-4; *B*, IL-10; *C*, TGF-β; *D*, IL-2; and *E*, IFN-γ. Lymphokines were not measured in the PP from IL-10 KO mice because these mice develop enterocolitis that interferes with the normal physiology of these lymphoid organs (45, 46).

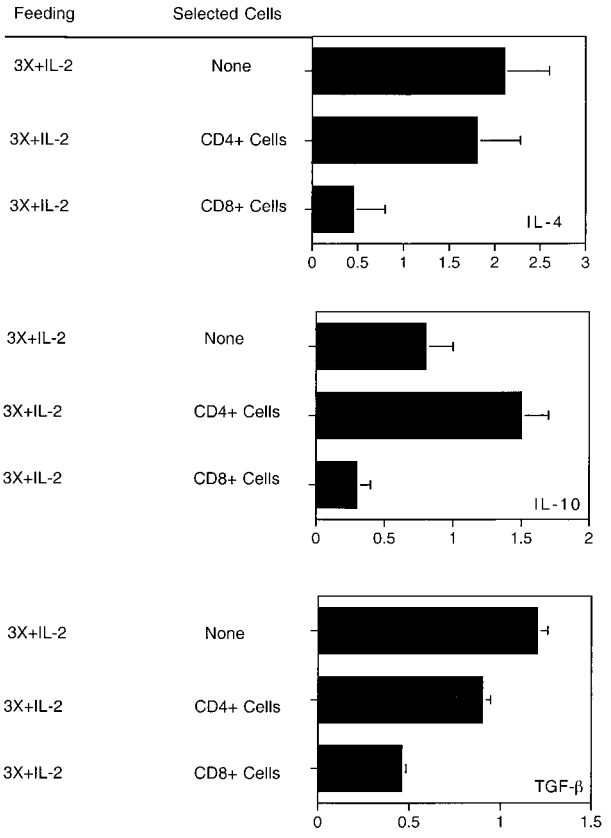

FIGURE 8. $CD4^+$ cells are responsible for most of the IL-4, IL-10, and TGF-β produced in PP after feeding with the 3× + IL-2 regimen. The wt C57BL/6 mice were fed either IRBP or PBS 3× + IL-2 as described in the text. Ten animals were used in each experimental group. All animals were immunized with a uveitogenic dose of IRBP (day 0). Twenty-one days after immunization mice were sacrificed, and PP were obtained. $CD4^+$ cells and $CD8^+$ cells were isolated by magnetic cell sorting as described in *Materials and Methods*. Cytokine production was evaluated in the culture supernatants as described in the text.

as we had previously reported in another system (35). PP were not evaluated in the IL-10 KO animals because the inflammatory bowel disease they develop impedes correct interpretation of the results. This finding suggests that IL-4, although crucial to the development of protection by the 3× + IL-2 regimen, is not associated with the lack of protection observed in the IL-10-deficient animals.

IL-10 synthesis was not detected in the IL-10 KO animals, and as shown previously, neither of the oral tolerance regimens was able to induce IRBP-specific IL-10 synthesis in the LN of treated animals (Fig. 7*B*). There were no differences in IL-10 production in PP between wt mice and IL-4 KO, confirming our previous results. Interestingly, IL-10 production was significantly decreased in the spleen of IL-4 KO mice compared with that in wt animals. The significance of this finding is hard to gauge, since in all other organs evaluated there appears to be no difference between the IL-4 KO mice and wt mice regarding IRBP-specific IL-10 production. Nevertheless, it is possible that the lack of IL-4 results in a decrease in a specific IL-10-producing cell population that, at the time we evaluated cytokine production, is predominately located in the spleen, and this population may be important for the development of oral tolerance. Adoptive transfer experiments are under way to clarify this question.

We have shown that the administration of IL-2 can potentiate the tolerogenic effect of oral administration of Ag (20). IL-2 was also shown to be pivotal to the maintenance of tolerance in naive animals, since IL-2-deficient mice develop spontaneous autoimmunity (36). Consequently, we decided to measure IL-2 synthesis in response to IRBP stimulation in the KO mice and their normal littermates. Interestingly, IL-10 KO mice synthesized lower levels of IL-2 than their normal littermates. IL-2 production in the IL-10 KO was undetectable in the spleen and LN of those mice that received either one of the tolerogenic regimens. As was the case for TGF-β, the different lymphoid organs showed different cytokine patterns; this lack of a consistent cytokine profile among spleen, LN, and PP suggests that different populations of Ag-specific T cell are present in these organs at the time we analyzed them for cytokine production. The decreased IL-2 production by IL-10 KO mice may be linked to the diminished proliferative response to IRBP seen in the LN of these mice. The pattern of IFN-γ production followed the same observed for IL-2 (Fig. 7*E*). We observed no difference between the groups regarding their production of IL-5, and TNF profiles were unremarkable among the different strains of mice (data not shown).

Many reports in the literature have suggested that TGF-β plays a pivotal role in the development of oral tolerance mediated by cytokines (37–39). As we had previously shown (20), TGF-β synthesis is enhanced in wt animals after feeding, and the 3× + IL-2 regimen induces higher levels of TGF-β than the 5× regimen (Fig. 7*C*). Interestingly, TGF-β expression was absent in the LN of IL-4 or IL-10 KO mice. Nevertheless, IRBP-specific TGF-β synthesis in cytokine-deficient mice was equivalent to that in wt animals in both spleen and PP. One could speculate that the decrease in TGF-β production is associated with the lack of induction of oral tolerance to IRBP in the cytokine-deficient mice.

Because some concern was raised regarding the source of the cytokines measured in response to IRBP, we isolated $CD4^+$ cells and $CD8^+$ cells from the PP of mice fed the 3× + IL-2 regimen. The results show that when $CD4^+$ PP cells are stimulated with IRBP in vitro, the synthesis of IL-4, IL-10, and TGF-β is similar to that observed when the whole cell population from PP is used. In contrast, when $CD8^+$ cells are stimulated with IRBP, there is a significant decrease in the synthesis of all cytokines analyzed (Fig. 8). These data suggest that $CD4^+$ cells are responsible for most of the IL-4, IL-10 and TGF-β synthesis observed after feeding the 3× + IL-2 regimen to mice.

Because TGF-β production in response to IRBP was diminished in the KO mice, we decided to treat these animals with recombinant TGF-β in an attempt to restore the protection induced by the 3× + IL-2 feeding regimen. We followed the protocol described previously (40). Equal numbers of IL-10- and IL-4-deficient mice were used in each group. Because the results for the two KO stains were not different, the combined data are presented in Fig. 9. The huge costs involved in obtaining the amounts of TGF-β necessary to perform these experiments forced us to limit the number of mice to three in some of the TGF-β-treated groups. Reconstitution was ineffective in restoring the effectiveness of the 3× + IL-2 feeding regimen in these animals (Fig. 9*A*). Furthermore, unlike the IL-10-treated mice, wild-type unfed mice treated with TGF-β were not protected against the development of EAU, confirming our previously published data (40) and data from a manuscript in preparation (H. Xu, L.V.R., P. B. Silver, and R.R.C.). Because of the small number of animals used in these experiments, we decide to take another approach to study the role of TGF-β in the deficient induction of tolerance in the KO mice. We treated these animals with a polyclonal Ab raised against TGF-β in chickens, using doses previously described as effective in neutralizing TGF-β in vivo. Our results suggest that Ab-treated wt animals are still capable of responding to the 3× + IL-2 regimen with induction of

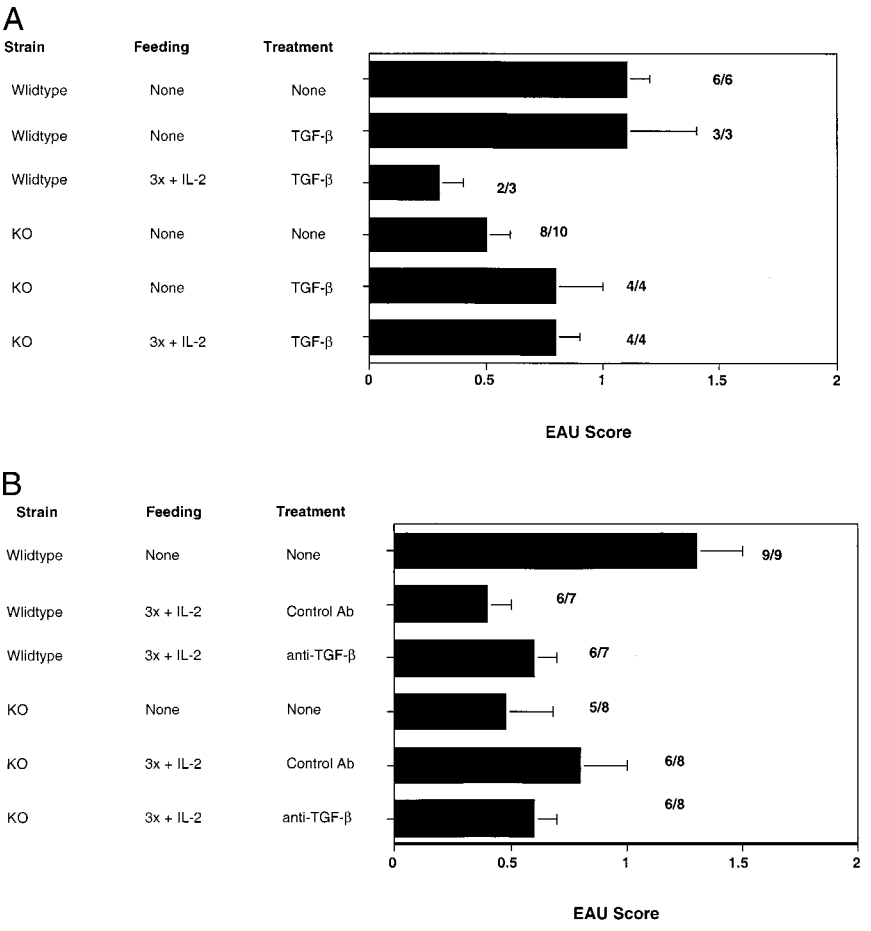

FIGURE 9. TGF-β treatment or anti-TGF-β treatment does not alter the course of oral tolerance induction by the 3× + IL-2 regimen. IL-4 and IL-10 KO mice and their normal littermates were treated with rTGF-β (*A*) in association, or not, with the 3× + IL-2 regimen of oral tolerance induction. Disease scores were evaluated 21 days after a uveitogenic dose of IRBP. In *B*, animals were treated with a polyclonal Ab against TGF-β in association, or not, with the same tolerogenic regimen, and disease was graded 21 days after immunization with a uveitogenic dose of IRBP.

tolerance to (Fig. 9*B*). Furthermore, KO mice treated with the anti-TGF-β Ab were not less susceptible to tolerance induction than animals treated with the control Ab. These data indicate that if TGF-β plays a role in oral tolerance to EAU, it is probably a minor one.

Discussion

The finding that both IL-4 and IL-10 seem to be required for induction of protective tolerance was unexpected. It is possible that IL-4 and IL-10 act synergistically in the tolerance induction process, as they do to inhibit delayed-type hypersensitivity reactions, and both cytokines are needed to induce optimal tolerance. Another explanation would be a requirement for the two cytokines to act at different stages of the tolerance process. We hypothesized that IL-4 is necessary as the growth factor for the regulatory cells induced by oral tolerization (27). IL-10, on the other hand, may be required as an effector cytokine to promote down-regulation of the inflammatory process. This last possibility is supported by our finding that IL-10 inhibits Ag-specific proliferation and IFN-γ production of murine Th1-like uveitogenic effector T cells in vitro (41).

A dissociation between lymphocyte proliferation to IRBP and disease status was found. Although the cytokine-deficient mice were not protected against the development of EAU by the 3× + IL-2 regimen, lymphocyte proliferation in response to IRBP was significantly decreased in the LN of the IRBP-fed mice compared with that in the OVA-fed controls. A dissociation between disease status and Ag-specific proliferation has been described previously (30, 31). It has been suggested that lymphocyte proliferation is not a good indicator of the presence or the activity of pathogenic cells (30, 31). Because we induce disease with IRBP of heterologous nature (bovine), different antigenic epitopes and cell populations might be involved in the two responses (proliferation and uveitogenicity); while pathogenic cells will respond to autologous epitopes, lymphocyte proliferation in the draining LN will be mostly due to cells responding to the nonself (bovine) epitopes of IRBP. This possibility would account for the differences between our findings and those reported previously that suggested that neither IL-4 nor IL-10 is required for the induction of oral tolerance (29, 42). In these studies animals were fed and subsequently immunized with exogenous Ags such as OVA, and proliferation and Ab production against these Ags were used as markers for tolerance induction. Because of the nature of the Ags used in these studies, the responses against their homologous counterparts, for instance murine albumin, could not be measured. Another possible explanation for the dissociation between lymphocyte proliferation and disease scores is a difference in the timing of the responses measured. Proliferation is assayed at the time that pathology is already fully developed, and most of the cells involved in the pathogenic response have migrated out of the draining LN. It is possible that all these factors may act in unison contributing to the dissociation between the proliferation results and EAU grade. Nevertheless, it is clear that even in the IL-deficient mice some degree of tolerance was induced by the 3× + IL-2 regimen, although it did not result in protection from the development of uveitis.

In keeping with our original observations (20), the 3× + IL-2 regimen induced IL-4, IL-10, and TGF-β production in the PP of normal mice. The 5× regimen was conspicuously less effective in inducing these cytokines. As before, we were unable to detect IL-10 production in the LN of wild-type or KO mice (20). In contrast to what was reported by others (43, 44), Ag-specific production of IFN-γ

by PP in our experiments was minimal. IFN-γ and IL-2 production in spleen and LN cells followed the pattern shown in the proliferative responses described above, i.e., were suppressed by both feeding regimens. Here it is important to notice that IL-2 production in the LN of IL-10 KO mice was diminished, and it may be either the cause or an effect of the diminished LN cell proliferation seen in response to IRBP on these mice. In contrast, in the PP, IL-2 was enhanced in response to the 3× + IL-2 regimen in both KO and wt mice. It is noteworthy that IL-4 KO mice had a decreased production of TGF-β. This finding is in line with a recent report that IL-4 acts as a growth factor for TGF-β-producing, mucosal-specific, regulatory T cells (27). The absence of IL-4 or IL-10 also seemed to result in a reduction of TGF-β secretion in the LN of fed animals and could be secondary to a decrease in the production of these cytokines in that organ. Thus, the need for IL-4 and IL-10 to elicit protective oral tolerance might be due in part to their effects on TGF-β production. To investigate this possibility and because TGF-β has been reported in the literature to be a major player in oral tolerance induction in other systems, we treated the KO mice and their normal littermates with recombinant TGF-β. The results showed that the treatment with the TGF-β we performed was ineffective in restoring the ability of the 3× + IL-2 feeding regimen to protect these animals against EAU. Although the doses and administration scheme we used proved to be effective in another system (40), it is possible that these doses are not adequate for the study of oral tolerance, and higher doses administered more frequently may be required to obtain any effect. However, the prohibitive costs of such experiments made us decide on alternative strategy. We treated animals with anti-TGF-β Ab. Several different combinations of doses and administration regimens were used. In these experiments we were limited by the fact that more than two inoculations of mice with chicken Ab resulted in serum sickness. The data we present are from a regimen we found to be most effective in diminishing IgA Ab synthesis, which we used as a marker of the effectiveness of the Ab to neutralize TGF-β. The wt animals remained sensitive to the induction of tolerance by the 3× + IL-2 regimen when treated with anti-TGF-β Ab. These data combined with the results of the TGF-β treatment of KO mice suggest that TGF-β plays a minor role in our system compared with the major role it plays in EAE, for instance. The reason(s) for such difference is unclear at this point.

It is possible that under the conditions we described, cytokines such as IL-4, IL-10, and TGF-β are being secreted by cells other than lymphocytes, thus explaining why tolerance against disease development is induced but cytokine synthesis remains present in response to retinal Ags. However, when $CD4^+$ cells were depleted from the PP, the secretion of IL-4, IL-10, and TGF-β was significantly decreased. These data suggest that $CD4^+$ cells are indeed involved in the production of such cytokines. In contrast, depletion of $CD8^+$ cells did not alter the expression of the same cytokines, except for a slight enhancement of IL-10 synthesis, probably due to a decrease in IFN-γ production. Although it may be argued that the 3× + IL-2 regimen does not induce tolerance toward IRBP but, rather, a shift in the type of cells that respond to that Ag, we and others (9–13, 15, 16) believe that the definition of tolerance goes beyond the inability to respond to an Ag. Particularly for autoantigens, the definition of tolerance must be more flexible, as demonstrated by the dissociation among disease scores, lymphocyte proliferation, and cytokine production we reported here.

Interestingly, both IL-4 and IL-10 KO mice had lower EAU scores than their wt littermates. Although mice in the C57BL/6 background are only moderately susceptible to EAU, the disease scores obtained with the KO animals were even lower. In the case of the IL-10 KO mice, we suspect that their frail physical condition, caused by the inflammatory bowel disease they develop over time, may have contributed to the diminished scores. This prediction is supported by the fact that mice treated with anti-IL-10 Ab were not less susceptible to disease induction than animals treated with a control Ab (Fig. 4). The lack of IL-4, however, does seem to affect disease development, since both the IL-4 KO mice and mice treated with the anti-IL-4 Ab 11.B11 develop lower EAU scores (Figs. 2 and 5). We have indications that small amounts of IL-4 may be required for the synthesis of IFN-γ (B. Sun, S. H. Sun, P. B. Silver, L.V.R., and R.R.C., manuscript in preparation). Although the role of IL-4 in the development of oral tolerance is undeniable since we were unable to tolerize the IL-4 KO mice with the 3× + IL-2 regimen, its precise importance remains to be determined, since the effect of anti-IL-4 treatment on oral tolerance was not significant. The discrepancy between the results obtained with the KO mice, which lack IL-4 from birth, and those obtained with anti-IL-4-treated animals may be due to the fact that precursor cells require IL-4 to develop, and the complete absence of this cytokine will impair their development. Whatever the case may be, it seems that a certain amount of IL-4 may be needed to induce EAU to its fullest extent.

In conclusion, the data presented here show that the ability to produce IL-10 is not required for disease induction in EAU. However, both IL-10 and IL-4 are required for the induction of protective oral tolerance through the 3× + IL-2 regimen that elicits anti-inflammatory cytokines, but not by the 5× regimen, which seems to be cytokine independent. The requirement for both IL-4 and IL-10 may result from a synergistic response or a sequential need for the two cytokines in inducing and effecting protection, with IL-4 acting at the induction stage and IL-10 at the effector stage. Ag-specific TGF-β production in response to feeding is decreased in mice deficient in IL-4 and IL-10 and may constitute a part of the mechanism, although neutralization of TGF-β or its replacement was unable to alter the course of disease in fed animals. A dissociation between tolerance measured by Ag-specific lymphocyte responses in culture and protection from EAU was observed. It may in part reflect responses to self vs nonself epitopes. The data reported here help to elucidate the mechanisms that drive oral tolerance and provide a rationale for exploring strategies that would enhance its protective effect through augmentation of IL-4 and IL-10 to maximize the clinical benefits of oral Ag therapy.

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